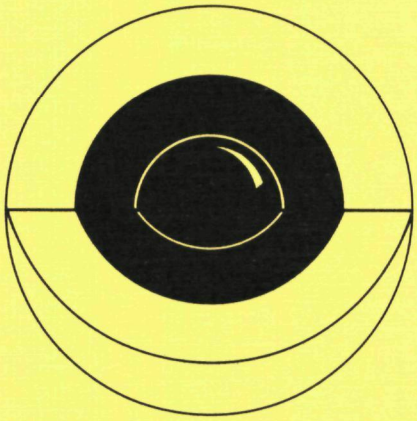
OXIDATIVE CHALLENGE TO AGING HUMAN LENS LEADING TO NUCLEAR CATARACT



P.M.M. van HAARD

TOELICHTING

Het doel van het hier beschreven onderzoek is een beter inzicht te krijgen in het ontstaan van cataract of staar in de ooglens van sommige ouder wordende mensen.

De lens stelt de mens in staat om scherp te zien. Een voorwaarde is wel dat de lens licht doorlaat. Elke verandering in de lichtdoorlaatbaarheid ten gevolge van troebel worden van de lens wordt cataract genoemd. Dit betekent niet dat onscherp zien altijd aan cataract te wijten zou zijn.

De lenstroebelingen, vóórkomend bij oudere mensen (90-95% van alle cataracten) worden ouderdomscataracten genoemd. Tot deze groep van cataracten behoort de kerncataract, het is deze vorm van cataract die ik onderzocht heb.

De kerncataract is om allerlei redenen een interessant onderzoekobject. Komen bij andere ouderdomscataracten witte troebelingen voor in verschillende delen van de lens, bij kerncataract wordt alleen het normaal lichtgeel gekleurde binnenste deel van de lens - de kern - meestal rond het vijftigste jaar steeds harder, troebel en bruiner van kleur. In het ernstigste en meest gevorderde stadium blijkt de harde, zwartbruine kern van de lens even groot te zijn als de lens bij de geboorte is.

Tot op heden blijkt de enige therapie te zijn een operatief ingrijpen, waarbij de lens verwijderd wordt en het gezichtsvermogen redelijk hersteld met een contactlens of een bril, voorzien van zeer dikke glazen. Het is logisch dat met toene-

mende medische controle eerder zal worden ingegrepen; er zullen in de toekomst ook minder zeer ernstige kerncataracten beschikbaar komen voor onderzoek.

De ogenschijnlijk onbeduidende bevinding dat de cataract groter wordt met het bruiner worden van de kleur, heeft belangrijke gevolgen gehad voor mijn onderzoek. Alvorens dit te kunnen uitleggen moeten we eerst meer weten over de ontwikkeling en veroudering van de lens.

De lens ontwikkelt zich voor de geboorte, aanvankelijk als een rond bolletje, de embryonale lens, waaromheen zich langgerekte cellen vormen, enigszins vergelijkbaar met de rokken van een ui. Het resultaat van deze ontwikkeling is dat de buitenlaag van de lens - de cortex - jonger is dan het binnenste gedeelte, de kern. Op elk moment in het leven van de mens is het binnenste deel van de lens even oud als de mens zelf.

Om alle cellen van de lens bij elkaar te houden, zit er om de lens een elastisch vlies, het kapsel, en via dit omhulsel is de lens verbonden met spieren die de lens van vorm doen veranderen; dit proces wordt accommodatie genoemd.

De lenscellen zitten boordevol eiwitten, die zo dicht en toch ook weer zo geordend gestapeld zijn dat elke cel in staat is licht dat erop valt van richting te doen veranderen; dit proces wordt lichtbreking genoemd. Accommodatie en lichtbreking zorgen voor scherp-zien.
Via het kapsel krijgt de lens voedingsstoffen toegevoerd, uit een met vocht gevulde ruimte voor de lens, de voorste oogkamer.

Een eiwit bestaat uit onderling verschillende bouwstenen, de aminozuren, die de vorm, grootte en andere eigenschappen en daarmee de funktie van het eiwit bepalen. De funktie van de lenseiwitten is vrij duidelijk: ze moeten struktuur geven aan de lenscellen en daarmee dus aan de lens. Over het ouder worden van deze eiwitten, van het celomhulsel (membraan genoemd), over stoffen in de lens die de bestanddelen van de lens een leven lang beschermen of daarin tekort schieten, en over andere mogelijke oorzaken voor het ontstaan van kerncataract handelt dit proefschrift.

Aangezien de lens een leven lang licht moet doorlaten, is het aannemelijk dat zonlicht een oorzaak kan zijn voor het ontstaan van kerncataract. Niet alle zonlicht is schadelijk voor de lens, het is mogelijk dat brildragers of binnenshuis werkenden beter beschermd zijn dan andere mensen die het moeten hebben van de lagen rond de aarde en de bescherming door het hoornvlies tegen schadelijk licht van de zon.
Het valt op dat op plaatsen waar de mens bloot gesteld is aan vele zonneuren (Thailand, Pakistan, Cambodja) meer kerncataracten voorkomen dan in onze streken. De vraag rijst echter waarom kerncataract ook in zonnige streken pas op hogere leeftijd optreedt en waarom niet overal in gelijke mate.

De lens groeit gedurende het hele leven binnen het kapsel: de buitenste delen (jong materiaal) worden tegen de gevolgen van schadelijk licht hoofdzakelijk beschermd door bepaalde eiwitten, enzymen, terwijl de binnenste lagen bijna uitsluitend

beschermd worden door stoffen die vrijer kunnen bewegen dan eiwitten.

Uit mijn onderzoek bleek dat in de binnenste delen van de lens de concentratie van die vrije stoffen afneemt naarmate men ouder wordt en dat die afname schrikbarend groot is in lenzen met kerncataract. Ik heb alleen onderzoek kunnen doen aan lenzen uit Duitsland en Nederland, wanneer men afgaat op de kleur van deze cataracteuze lenzen en die uit Pakistan blijkt dat mensen in Pakistan op jongere leeftijd kerncataract krijgen dan in onze streken. De faktor zonlicht speelt hier vermoedelijk een rol.

In het kort komt het er vervolgens op neer dat er met de afname van de concentratie van beschermende stoffen beschadigingen optreden in de eiwitten van de binnenste cellen van de lens. Het proces van verkleuring en troebeling start daarom in het oudste deel van de lens: de embryonale lenskern en spreidt zich uit met het donker worden van de kleur, naar buiten toe.

De termen die voorkomen in de titel van dit proefschrift worden hiermee al een stuk duidelijker: "challenge" betekent in eerste instantie "uitdaging" en met de toevoegingen "oxidative" en "aging" wil ik aanduiden dat die uitdaging op den duur tot een beschadiging van verouderde lenscomponenten ten gevolge van een oxidatieproces leidt. Nu kan zuurstof niet verantwoordelijk zijn voor deze oxidatie, omdat het praktisch niet voorkomt in de lens. Het zoeken naar stoffen die hetzelfde ef-

fekt kunnen hebben als zuurstof werd versneld door bepaalde bevindingen in dit promotieonderzoek waarbij, afgaande op de struktuur van oxidatieprodukten, kon worden afgeleid dat waterstofperoxide een rol zou kunnen spelen bij het ontstaan van kerncataract. De vraag is daarmee niet opgelost, want wij moeten nog te weten komen waar dat waterstofperoxide vandaan komt.

Wij veronderstellen dat een bepaalde bouwsteen van de lenseiwitten lichtenergie in zich opneemt en daarbij niet in brokstukken uiteenvalt, maar die energie overdraagt aan natuurlijke beschermers die aanvankelijk in overvloed aanwezig zijn. Langzamerhand verdwijnen deze beschermende stoffen bij het vervullen van hun taak, en zo zal op den duur energie worden overgedragen aan eiwit en andere bouwstenen van de lens, met schadelijke gevolgen voor de lens en de eiwitten daarin.

Dit proces treedt in de lens bij hoge leeftijd op. De eiwitten klonteren samen, hetgeen tot troebeling en verkleuring leidt. Het samenklonteren van de eiwitten heeft mogelijk tot gevolg dat de celmembranen van struktuur veranderen zonder dat deze zelf door zonlicht etc. zijn aangetast; de aanvankelijk soepele membranen van de lenscellen ondergaan wel - tijdens veroudering - een aantal veranderingen in samenstelling, die het hele cataracteuze proces kunnen versnellen.

De oorzaak van de verkleuring van de lenskern heb ik maar ten dele kunnen ophelderen, aangezien het onderzoeken van onontwarbaar materiaal speciale technieken vereist die duidelijk tekortkomingen bezitten. Wanneer je met chemische middelen niet verder kunt doordringen in de materie, kun je nog afbre-

kende stoffen (bepaalde enzymen) gebruiken die door de natuur ontworpen zijn om alleen natuurlijke stoffen aan te vallen (of er nuttige stoffen van te maken). Blijkbaar zijn de facetten van kerncataract niet natuurlijk en zijn de samengeklonterde eiwitten uit de lens te vergelijken met zwarte, zich opstapelende stoffen die ook voorkomen in andere, langlevende, cellen van het menselijk lichaam, zoals de hersencellen.

Een tweede belangrijk punt bij het beschrijven van kerncataract en de moeilijkheden van onderzoek daaraan is dat, tijdens kerncataract, de normale veroudering van de lens doorgaat met zijn eigen specifieke verschijnselen. Het is dus onjuist te veronderstellen dat kerncataract een vorm van versnelde veroudering is: de verschijnselen die optreden bij kerncataract zijn ten dele te vergelijken met normale veroudering, omdat normale veroudering kan leiden tot kerncataract, afhankelijk van invloeden van buitenaf, bijvoorbeeld zonlicht.

Kerncataract zou teruggedrongen kunnen worden, al moeten we wel minstens 40 jaar op de resultaten wachten, totdat de mens in zonrijke streken beter voedsel beschikbaar had en zijn ogen voortdurend kon beschermen tegen zonlicht. Wij zullen ook in dit opzicht zuinig moeten zijn op de beschermende ozonlaag rond de aarde die steeds meer aangetast lijkt te worden door uitlaatgassen van vliegtuigen en drijfgassen uit spuitbussen.

Nijmegen, 18 september 1980 P.M.M. van Haaro

OXIDATIVE CHALLENGE TO AGING HUMAN LENS LEADING TO NUCLEAR CATARACT

Promotores: Prof. Dr. H.J. Hoenders
Prof. Dr. H. Bloemendal
Coreferent: Dr. J. Haverkamp

OXIDATIVE CHALLENGE TO AGING HUMAN LENS LEADING TO NUCLEAR CATARACT

PROEFSCHRIFT

ter verkrijging van de graad van doctor
in de wiskunde en natuurwetenschappen
aan de Katholieke Universiteit te Nijmegen
op gezag van de Rector Magnificus,
Prof Dr P G A B Wijdeveld,
volgens besluit van het College van Decanen
in het openbaar te verdedigen
op donderdag 18 september 1980,
des namiddags te 2 00 uur precies

door

PAULUS MARIA MATHIAS VAN HAARD

geboren te Heerlen

Krips Repro Meppel

1980

Belonging to someone I find is necessary
The load is lighter on your mind
when someone helps to carry
And even though I'm strong enough
to make it on my own
I would not even care to try
to live my life alone

David Gates

Aan Henny en mijn moeder

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CONTENTS

A/F	: Fluorescence, Excitation wavelength/emission wavelength
α	: Level of significance
Br	: Brown nucleus
BSTFA	: Bis-silyl trifluoroacetamide
Co	: Cortex
D	: Daltons
Db	: Dark brown nucleus
d.f.	: Degrees of freedom
DNS-Cl	: 5-Dimethyl amino naphthalene-1-sulfonic acid chloride
DTE	: 1,4-Dithioerythritol
DTNB	: 5,5'-Dithio (2-nitro-benzoic acid)
EDTA	: Ethylene diamino tetraacetate
ENu	: Embryonic nucleus
g	: Gravitational acceleration (981 cm sec^{-2})
GC	: Gas chromatography
GSH	: Glutathione
h	: Hour(s)
High-M_r	: High molecular weight
HPLC	: High pressure (performance) liquid chromatography
i.d.	: Inner diameter
IEF	: Isoelectric focusing
k	: Capacity factor (ratio)
λ	: Wavelength

MeOH : Methanol

min : Minute(s)

M_r : Relative molecular mass

MS : Mass spectrometry

nm : Nanometer

n : Number of determinations

nd : Not determined

NaOAc : Sodium acetate

Ni : Nigra nucleus

No : Normal nucleus, lens

NP- : Non-protein-bound

Nu : Nucleus

P : Probability

Pa : Pascal (1 atm = 101,25 kPa)

P- : Protein-bound

Py-MS : Pyrolysis mass spectrometry

R_f : Relative mobility

RP : Reversed phase

RT : Retention time

SH : Sulfhydryl

Si : Silica

S.D. : Standard deviation

SDS : Sodium dodecyl sulfate

SS : Disulfide

TCA : Trichloro acetic acid

Tr : Trace

Tris : Tris (hydroxymethyl) amino methane

UV	: Ultraviolet
VAC	: Very acidic chains
WI	: Water-insoluble
WS	: Water-soluble
Ye	: Yellow nucleus
US	: Urea-soluble
UI	: Urea-insoluble
UDS	: Urea-DTE-soluble
UDI-E	: Urea-DTE-insoluble extracted

CHAPTER I

GENERAL ASPECTS OF THE HUMAN LENS 7

The mammalian lens is one of the most unique tissues of the body. It is an avascular, transparent, biconvex structure surrounded by an elastic capsule (Fig. 1). The prenatal lens, in contrast with the adult lens, is surrounded, during almost the whole of gestation, by a closely attached and rich network of capillaries revealing a vascular environment.

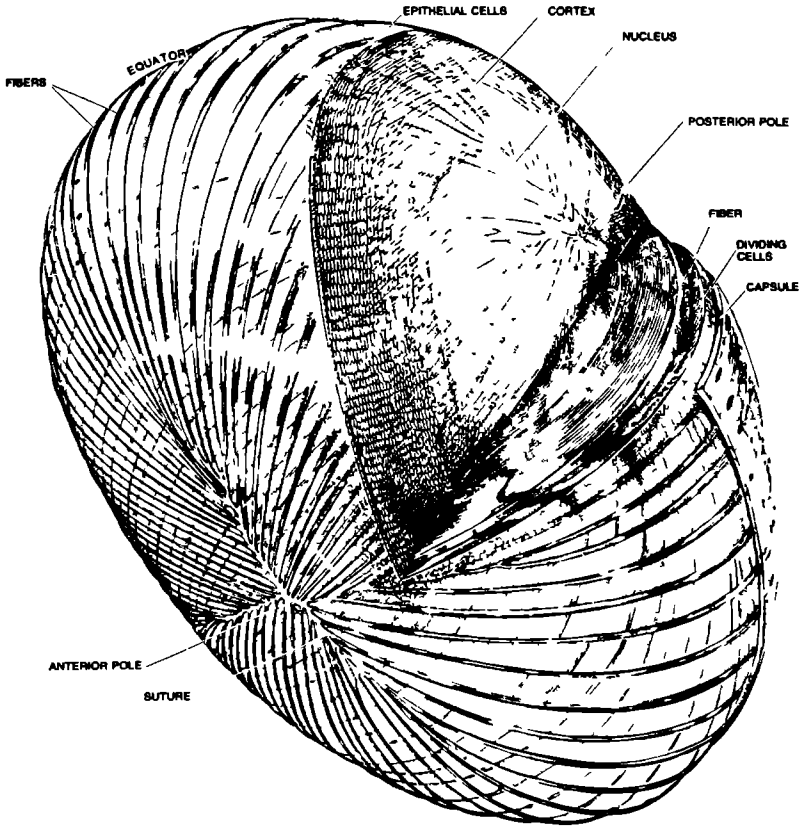


Fig. 1. From an "What happens to the human lens in cataract" by R. van Heyningen. Copyright (c) 1975 by Scientific American Inc. All rights reserved.

Until the fifth month of gestation no aqueous humor is produced, but after that time nutrients and oxygen can enter the lens through the capsule from the aqueous chamber and perhaps from the vitreous body. After the fifth month, the lens is isolated from the body's reticulo-endothelial and circulatory system and, as a result, the proteins of the lens are strongly antigenic. The lens gains most of its energy supply from glycolysis.

The lens is suspended by zonular fibers from a coronary muscle between the aqueous chamber and the anterior face of the vitreous body. The primary function of the lens is to refract and focus light on the retina, thereby producing a sharp image. In order to accomplish this the lens must remain metabolically active, transparent and be able to accommodate. For still unknown reasons some mammalian lenses, mostly from diurnally active animals, are colored.

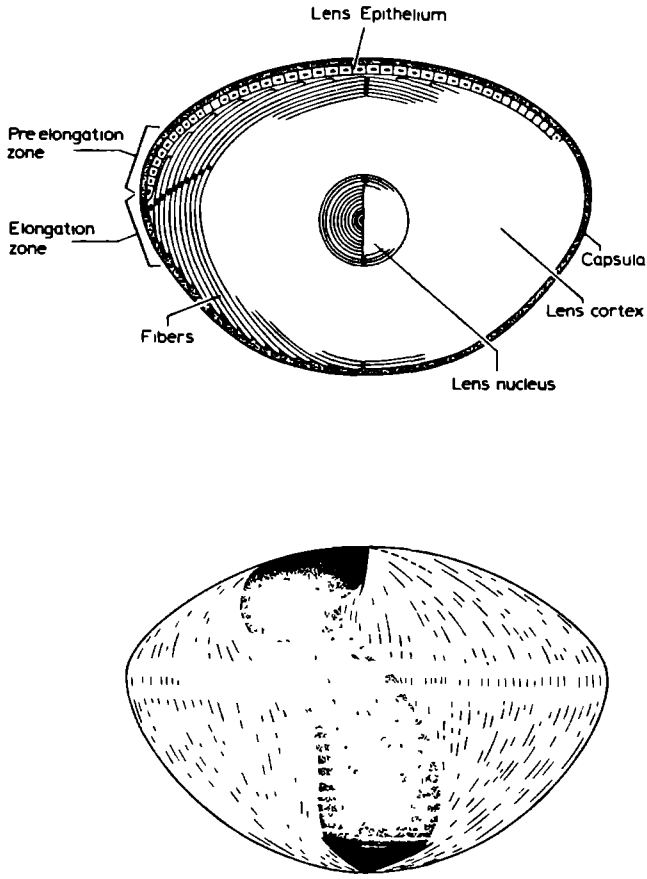
Deviations from normal lens transparency are called opacities or cataracts. The word cataract is derived from the Greek noun KATARAKT which means "waterfall". In the old days, people believed that cataract was an accumulation of water (humor) and the lens a "drop of humor" in which the faculty of sight was seated. When lenses were removed with a hollow needle, as the Arabian ophthalmologists did, lens mass gushed out like water.

The process of accommodation, i.e. light focusing, is accomplished through relaxation of tension of the zonular fibers upon contraction of the coronary ciliary muscle. In

some mammals a different mechanism of light focusing is known by which the lens is moved forward and backward. During the process of accommodation occurring in most mammals, lens shape and deformability are determined by the elasticity of the lens capsule and the lens cells. The elastic properties of the capsule reside in the coiled collagen filaments. The anatomic basis for the elastic properties of the lens cells seems to reside in a well-defined structural organization, known as the cytoskeleton, composed of contractile proteins. The pattern of distribution of this skeleton appears to correlate with gross shape and accommodation ability of the mammalian lens.

On the anterior side of the lens, there is a monolayer of epithelial cells. The lens arises from this single type of cells, which on differentiation pass through an elongation zone and become fiber cells. During the elongation process, synthesis of proteins and cell membranes is rapid in order to keep up with the large increase in cell volume. Cells from the equatorial-posterior region of the lens elongate and wrap around the periphery of the lens in an anterior and posterior direction with a screw-shaped movement (Fig. 2). The formed fiber cells stop growing when they touch each other at the front and the back of the lens, forming Y-shaped boundaries, the sutures. During and shortly after elongation, most cell organelles are lost from the fiber cells: cells from the inner parts of the lens are essentially featureless and devoid of protein synthesis. The lens grows throughout life

Fig. 2



within the capsule, layering fiber cell on fiber cell around a central core. Taking a snapshot, in the outer region of the adult lens, the cortex, newly synthesized cells are found, whereas in the inner part of the lens, the nucleus, prenatal fiber cells are present, which are as old as the individual and have undergone the process of aging. The

normal human lens keeps soft throughout life, whereas lenses from cow, rat and rabbit reveal a very hard nucleus. The shape of the fiber cells changes going from cortex to nucleus. Originally they are hexagonal in cross-section and reveal boundaries formed out of complicated systems of interdigitations. Fusion of adjoining fiber cell membranes into junctions occurs, enabling the lens cells to communicate with each other.

The protein content of the lens is higher than that of any other mammalian tissue. Most of the lens proteins are water-soluble. Although individual proteins and membranes can form turbid suspensions *in vitro*, the pool of lens proteins, plasma membranes and other lens components build up a tissue which is completely transparent to light. Individual proteins of the lens are synthesized in different parts of the epithelium, multi-subunit proteins may be not synthesized at once. Cell synthesis does not always occur at the same rate throughout the growth of the lens, giving rise to a changing refractive index, which is a function of protein concentration, within concentric layers of the lens: the discontinuity zones. Lens proteins are not renewed, that is, broken down completely and their components reutilized, as is usual in other mammalian tissues.

As far as the biochemist is concerned, the lens represents a model system in which post-translational alterations in proteins and membranes can be studied. Changes in the structural proteins of the mammalian lens during aging and cataractogenesis

have been reviewed by Harding and Dilley (1976). The reader is further referred to Barber (1973), Bellows (1975), van Heyningen (1975) and Bloemendal (1977) for detailed information about the lens and cataractogenesis.

Lens opacities attack each year millions of people all over the world and cause visual disability; no therapy is known. At present, lenses are extracted in order to restore sight with spectacles or artifical lenses.

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1. GENERAL ASPECTS OF THE HUMAN LENS

1.1. Introduction

The lens may be studied at the following three levels: a) as a single organ, b) as a tissue composed of epithelial cells, fiber cells and intracellular space, and c) on the molecular level, including high molecular weight substances and interface membranes (Iwata, 1974). We shall discuss the aspects of the human lens with reference to this scheme.

When the lens is viewed at the first level, it is regarded as an isolated organ, suspended between two biophases, called the aqueous chamber and the vitreous body, which supply the lens with nutrients and oxygen. Its function is refraction of light and, perhaps to some extent, defending the vitreous and retina from damaging light. The second level is concerned with the lens as a clone of cells, which can only be studied *in vitro*. This level is also associated with membrane function and questions, how substances are transported from and excreted into the external environment. The third level is concerned with the lens' proteins and plasma membranes: changes in their synthesis, structure and composition are the subject of many investigations on the lens with respect to development, aging and cataract formation.

Lens opacification (cataract) is a major cause of human visual disability throughout the world (Caird et al., 1965; Spector, 1974; Braendstrup, 1977). The opacification process consists of many stages and patterns, to quote Iwata (1974):

the start of lens opacification appears to be composed of a biochemical remote cause and a physicochemical immediate cause.

In order to study the types of cataract, at any level, we need a classification scheme. Knowledge about the incidence of cataracts may help us to compose such a scheme; moreover, it may teach us more about the possible causes leading to the development of senile nuclear cataract which is the subject of this thesis. In the following sections we shall try to find answers to these questions.

1.1.1. Classification of cataracts

Cataracts can be roughly divided into experimental (including accidental) and pathological (including congenital and age-dependently occurring) types. Toxic substances, metabolic disorders, heat, intraocular pressure and ionizing radiation, but also medicine, administered for diseases not concerning the eye, may cause several types of lens opacifications and pigmentations (detailed information is given by Bellows, 1975).

The advent of slitlamp biomicroscopy, nowadays perfectioned with photographic equipment, working, for example, according to Scheimpflug's principle (Dragomirescu et al., 1978), enables the ophthalmologist to study the living eye, to localize lens opacities and to estimate their dimensions even in a 3-dimensional way. Using the slitlamp a diagonally cross-section of the lens is obtained.

Most human cataracts occur at the age over 50 years. Primary age-related opacities in the human lens (constituting 90-95% of all cataracts removed) were included in the past into a single category known as "senile cataract". Within this category, human cataracts were classified *in vivo* and/or *in vitro* on the basis of location of opacity (Mach, 1963; 1964; Maraini and Pescatori, 1972; Nordmann, 1972; Friedburg, 1973), of increasing nuclear pigmentation (Pirie, 1968; van Heyningen, 1972a) or only by age (Clark et al., 1969). The discussion is still going on, but for the present the Pirie-van Heyningen classification system is generally used. It has been refined by spectroscopic evaluation performed *in vitro* on intact lenses (Lerman and Borkman, 1976) and decapsulated lenses (Lerman et al., 1978) and by determinations of cation contents (Duncan and Bushell, 1975; Duncan and van Heyningen, 1977; Bushell and Duncan, 1978). The latter three methods are time-consuming.

A second system (Chylack, 1978), relying on photographic reproduction of slitlamp biomicroscopic pictures, seems to be very suitable to both surgeons and cataract investigators; this system is still complex, since it describes each individual lens, using number scales for opacity and color. Detailed information about our classification system, based on cataract location, intensity of opacity, color and age will be given in Chapter II.

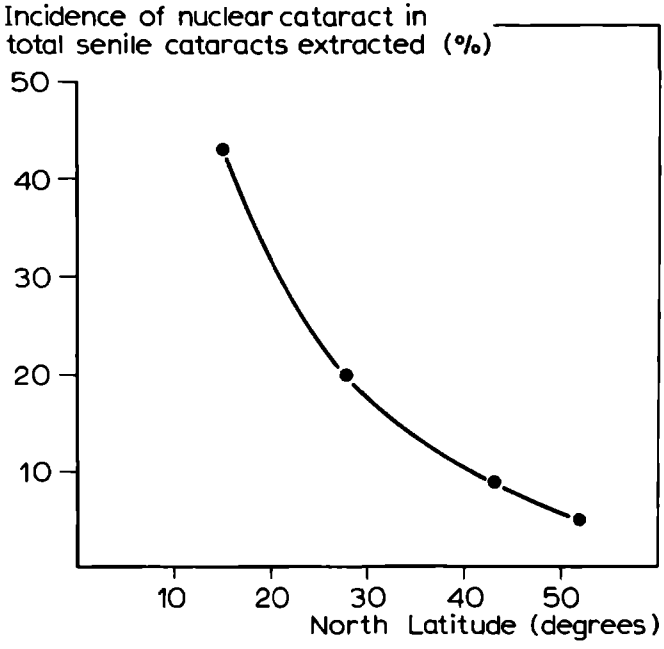
1.1.2. Incidence and putative causes of senile nuclear cataract

Cataract incidence in different geographic locations has been studied by several investigators (Duke-Elder, 1940; Fuchs, 1960; Halevi and Landau, 1962; Pirie, 1972a; Chatterjee, 1973; van Heyningen, 1975; Hiller, 1977; Zigman et al., 1979). Although cataracts are very common over the world, in areas where the yearly exposure to sunlight is high (India, Pakistan, Thailand) cataracts may develop early and with high incidence. On the other hand, cataracts are not found throughout the screened areas and are not prevalent at high altitudes. The presence and quality of water and food, the patient's occupation (outdoor-indoor) and racial differences may be important factors. The role of each factor is not fully understood. At all ages, more cataracts occur in Israel than in England (Pirie, 1972a; van Heyningen, 1975), less in Sweden than in Thailand (Halevi and Landau, 1962). In Pakistan, three times as many brown (nuclear) cataracts were extracted than in England (Pirie, 1972a). To date, no information is given in literature on cataract incidence of individuals, who at variable intervals emigrated from sunny areas to temperate climates or vice versa, as pointed at by Chatterjee (1973).

In a recent report (Zigman et al., 1979), the incidence of cataracts has been studied at different latitudes on earth, taking into account the yearly exposure of patients to sunlight (Johnson et al., 1976). Eighty percent of all

patients at every latitude were between 55 and 75 years of age. Although no full description was given, it was shown that the incidence of brown (nuclear) cataracts followed an obvious pattern whereas that of other cataracts did not. The percentage participation of brown (nuclear) cataracts at (estimated) 52^{o} North Latitude has been determined by Pau (1976). The available data are depicted in Figure 1.1. Determinations were performed in Manilla, The Philippines (133 lenses), Tampa, Florida (90 lenses), Rochester, New York (200 lenses) and Germany (1000 lenses).

Fig. 1.1.



A role of sunlight in senile nuclear cataract development in human lens has been suspected for many years (for review,

cf. Zigman, 1977; Lerman, 1976; Lerman and Borkman, 1978a, b). It should be noted that nuclear cataract has never been evoked experimentally in transparent lenses, either by (simulated) sunlight or by radiomimetic drugs (van Heyningen, 1976; Kuck, 1976a,b; Lerman and Borkman, 1979). Young human lenses (6 months to 8 years) transmit up to 75% of UV-light with wavelengths between 300 and 400 nm, being the only natural UV-light that can reach the earth through the ozone layer and that can reach the lens through the cornea and aqueous chamber (Bachem, 1956; Lerman and Borkman, 1976; Pitts et al., 1977; Zigman, 1977). The yearly exposure to light of wavelenghts > 300 nm depends on geographic location (Johnson et al., 1976), whereas spectacle wearers may be protected against light with wavelengths <350 nm (non-prescriptional crown glass, 2 mm thick; Zigman, 1978).

Above 25 years of age transmission by the lens of light with wavelengths between 300 and 400 nm drops to 20%, in aging human lens a progressively greater intensity of "blue fluorescence", yellow coloration and increasing pigmentation occurs, leading ultimately to nuclear cataract (Lerman, 1976; Lerman and Borkman, 1976; 1978a,b; 1979). Investigators still debate the reasons for the age-related increase in human lens pigmentation; two hypotheses have been put forward. The first is the *"growth hypothesis"*, stating that increasing lens pigment density is a simple consequence of lens growth (Mellerio, 1971; Coren and Girgus, 1972). In human lens several pigments should be considered, one of

which becomes less concentrated after birth, while others increase in amount during aging (Cooper and Robson, 1969; Lerman and Borkman, 1978b). Several pigments in human lens (kynurenine-glucosides) are formed metabolically and may function as a protective, 360 nm-light absorbing filter with respect to the vitreous body and the retina (van Heyningen, 1973a) or may inhibit color confusion in retinal cells (Dartnall, 1957). The second hypothesis is the *"ultraviolet hypothesis"*: increase in lens pigmentation is the result of UV-light exposure of lens components during lifetime (for review, cf. Zigman, 1977).

Several arguments have been raised against both hypotheses. It is well known that the major accumulation of lens pigment (yellow to black color) and fluorescence is found in the nucleus of the lens, not peripherally in the cortex. The human lens, like that of many diurnally active mammals, is yellow (Cooper and Robson, 1969; Wolbarsht, 1976), which fact might cause some confusion. The growth of the lens, according to the growth hypothesis, would result in darker pigmentation of the cortex, which does not actually occur in nuclear cataracts (Zigman, 1978). Against the UV-hypothesis arguments were raised (Dilley, 1975a) such as: examination of the nuclear part of nuclear-cataractous lenses had yielded no hard evidence of photo-oxidized proteins (van Heyningen, 1975); the destruction of the most photo-oxidation susceptible amino acid tryptophan, as found after *in vitro* irradiation of tryptophan-containing proteins (Buckingham and

Pirie, 1972; Holt et al., 1977), might lead to browning, cross-linking and insolubilization of proteins (for review, cf. Zigman, 1977), but no decrease in tryptophan content was found in nuclear lens proteins (Dilley and Pirie, 1974; Stein et al., 1976); proteins from the lens nucleus are no more susceptible to light than cortical proteins (Dilley and Pirie, 1974); in brown nuclear cataracts only the lens nucleus is pigmented, although most of the supposed damaging light would be absorbed at the front of the lens, if it were transmitted by the cornea (Dilley, 1975a); no evidence of increased lens pigmentation due to sunlight could be found, after lifetime extrapolation, when about 200 volunteers (18 to 25 years of age) from British Columbia, half wearing protective glasses, were tested after various periods of sunlight exposure, utilizing metameric color matching methods (Girgus et al., 1977). Men had stronger pigmented lenses than women (Girgus et al., 1977).

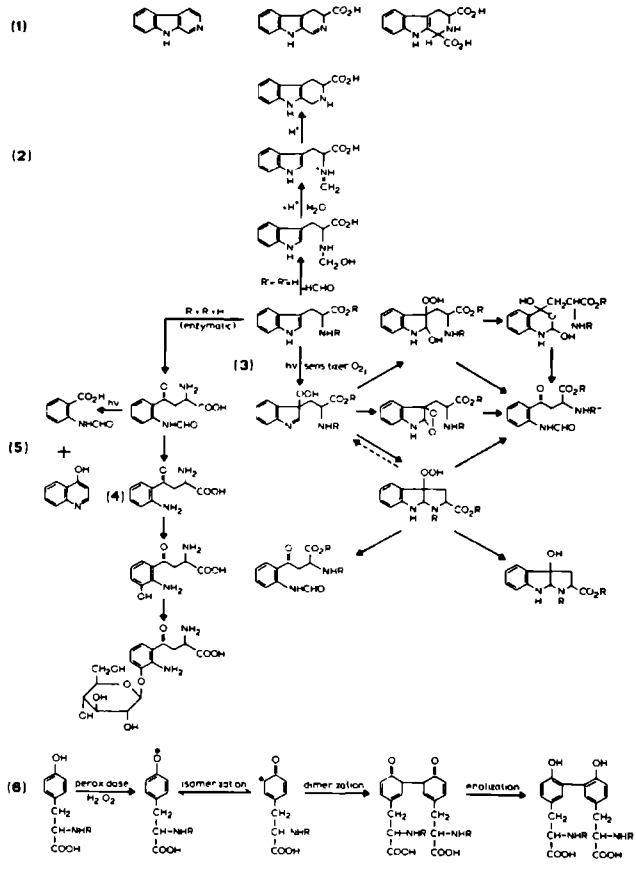
Some pigments in the human lens are light-sensitive, in particular to light of near-UV wavelengths (300-400 nm); others like N_1-formyl-kynurenine are near-UV-light sensitizers for lens proteins, that is, they absorb light of shorter wavelenghts and transduce the energy to other lens components (Pirie, 1971a,b; Pirie and Dilley, 1974; Walrant et al., 1975). At least two fluorophors, increasing in concentration upon aging of the lens, have been described; they were characterized by excitation/emission wavelength maxima (A/F) of 360-370/420-440 and 400-435/500-520 nm. Recently,

fluorophors have been found in calf lens proteins that were photoconvertible, that is, these fluorescent compounds were reversibly formed by light of 360 and 420 nm, respectively (Fujimori, 1979). A fluorophor, characterized by maximal excitation at 514,5 and emission at 556,4 nm, has been found in human lenses older than 23 years, using a laser Raman spectrometer as a fluorometer (Yu, 1977; Kuck and Yu, 1978). Fluorescence lifetime was determined in human lenses (Lerman and Borkman, 1978a,b), yielding at A/F 360/440 a value of 4,3 nsec; at pH 7.0 and 25°C, N_1-formyl-kynurenine yielded 1.3, anthranilic acid 3.9 and phenoxazines and β-carbolines more than 23 nsec.

The involvement of tryptophan in the process of browning of the human lens nuclear proteins during nuclear cataract development has been discussed by many investigators (for review, cf. Zigman, 1977; Weiter and Finch, 1975a,b; Lerman, 1976; Lerman and Borkman, 1978a,b; 1979). The original UV-hypothesis considered destruction of tryptophan residues in lens proteins as a main cause of yellowing and cross-linking of proteins (for review, cf. Kurzel et al., 1977), although strong evidence was missing. Arguments were substantiated referring to inactivation of enzymes or inhibition of cell division caused by photoproducts of tryptophan (Sun et al., 1979; Yoakum and Eisenstark, 1972; Walrant and Santus, 1974; Glatzer et al., 1976; McCormick et al., 1976; Zigman et al., 1976a,b; 1977; 1978). Some photoproducts of tryptophan are identified (Nakagawa et al., 1977; McCormick et al., 1976).

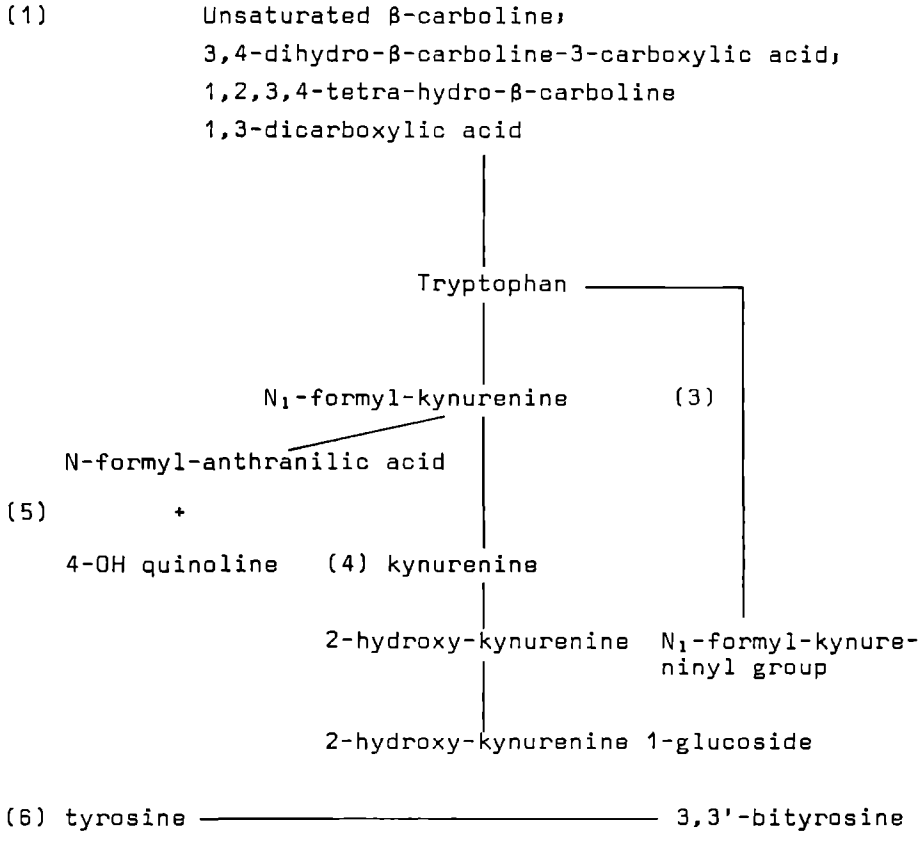
Mechanisms for tryptophan destruction by dye-sensitized photo-oxygenation have been described by the group of Witkop (cf. Nakagawa et al., 1977). Sun and Zigman (1979) proposed that the direct photo-oxidative pathway of near-UV-light exposed tryptophan follows a scheme, which is similar to the one described by Nakagawa et al. (1977). This scheme, including other information, is depicted in Figure 1.2:

Fig. 1.2.



McCormick et al. (1976) found that hydrogen peroxide was a photo-oxidation product of tryptophan and suggested that much of the damaging effect of photo-oxidized tryptophan might be explained by the action of hydrogen peroxide. Zigman et al. (1978) found differential effects of tryptophan photoproducts and hydrogen peroxide on the growth of mouse fibroblasts.

Fig. 1.2.

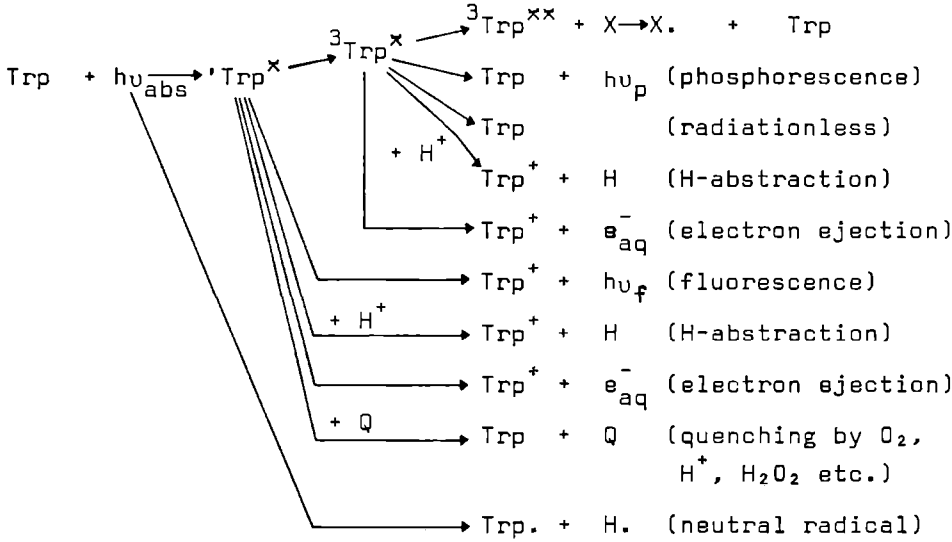


Before being destructed, tryptophan molecules are excited by the action of light. Comparing action spectra for tryptophan destruction in aqueous solution by UV-light (280 nm) with chromophore production spectra, it was concluded that photo-ionization of tryptophan might be the primary process in tryptophan photo-oxidation (Borkman, 1977; Borkman et al., 1977). According to Amouyal et al. (1979), however, the destruction of tryptophan in aqueous solution by near-UV-light (>300 nm) does not involve monophotonic ionization or electron ejection; moreover, below the ionization threshold energy, no primary ionization of tryptophan, giving inter alia Trp^+ (ionized tryptophan) and $O_2^{-\bullet}$ (superoxide anion radical), is required to account for the observed Trp· (tryptophan radical) and N_1-formyl-kynurenine production. The rate of fluorophor production out of tryptophan, which might be mainly N_1-formyl-kynurenine, showed little action at 360 and 320 nm irradiation, but increased sharply at 300 nm (Borkman et al., 1977a). Upon flash photolysis ($300<\lambda<360$ nm), a neutral radical (Trp·) was the only primary photoproduct recorded by Pailthorpe and Nicholls (1971); this radical might account for the production of $O_2^{-\bullet}$ (Amouyal et al., 1979). The photochemical attack on tryptophan has been summarized in scheme 1.1, utilizing literature data as given by Lerman (1976) and Amouyal et al. (1979). Secondary processes, tentatively assumed to occur in irradiated aqueous solutions of tryptophan are also depicted. The biology of oxygen radicals, which may occur in irradiated solutions of tryptophan, has

been reviewed by Feeney and Berman (1976) and Fridovich (1978). More aspects of radicals will be discussed in following sections.

Scheme 1.1. PHOTOCHEMICAL ATTACK ON TRYPTOPHAN

Primary processes:



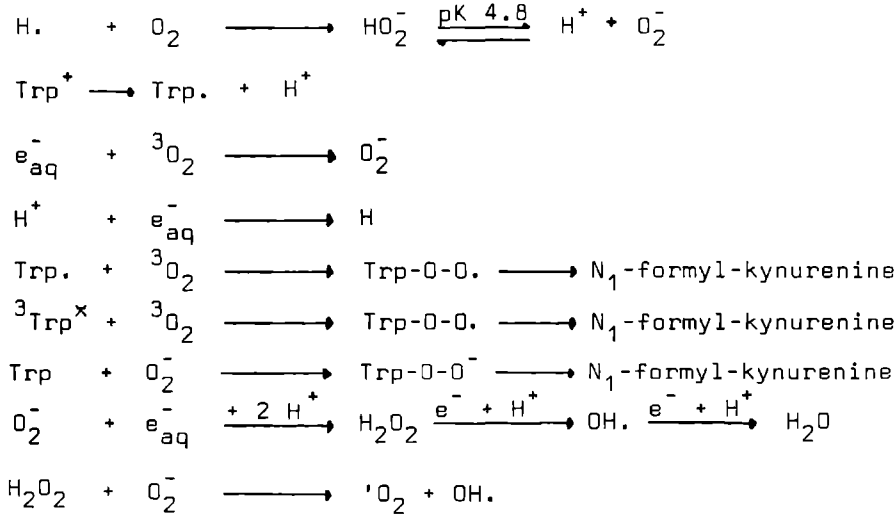
Addendum: electronic states of a molecule

A molecule is defined as a singlet if it has an even number of electrons, which are all arranged in pairs with opposite spins. The term spins denotes the fact that electrons have a magnetic moment pointing up or down.

A triplet is a molecule in which one pair of its even number of electrons reveals parallel spins.

A molecule with an odd number of electrons must have one electron with unpaired spin. This molecule is called a radical. The magnetic properties of this odd electron can be measured in the Electron Spin (paramagnetic) Resonance Spectrometer. Applying a strong magnetic field to the compound being studied, one can measure the energy absorbed when odd electrons flip their spins into an opposite direction. The amplitude of the energy peaks and their location at specific field strengths may be used to identify the nature and relative amount of the radical present in the sample.

Secondary processes:



Explanation of symbols:

x = excited state

xx = second excited state

3 = triplet state

1 = singlet state

Q = Quenching substances

Evidence for free radical mechanisms in fluorophor production in aging lens has been obtained by Borkman and Lerman (1977); ESR-signals from UV-light exposed lenses were found to be greater in young than in old human lens and greater in normal than in nuclear-cataractous lenses of the same age. The decrease in the rate of decay of photo-excited triplet states of tryptophan in lens proteins was nearly identical with the build-up rate of free radical signal (Yamanashi and Zuclich, 1978); a second photo-excited triplet species was found, which might be N_1-formyl-kynurenine.

Until recently, the UV-hypothesis seemed to be transformed into a free radical theory, according to which UV-light is the inducing factor and tryptophan, concomitantly with its destruction, the generating factor (Weiter and Finch, 1975a,b; Borkman and Lerman, 1977; Lerman and Borkman, 1978a,b). According to Weiter and Subramanian (1978), however, free radicals in human lens may be produced by a biphotonic process involving tryptophan, but not concomitantly with destruction. Radicals would then be produced and transmitted to other components according to a scheme, postulated by Siegal and Eisenthal (1965); this scheme has been depicted in scheme 1.1, first line.

The free radical hypothesis could explain many findings, such as yellowing and cross-linking of proteins, after irradiation with near-UV-light in the presence of tryptophan or kynurenine-glucosides (for review, cf. Zigman, 1977). It was observed that the rate of UV-light induced fluorophor product-

ion in human and animal lenses, both *in vivo* and *in vitro*, was greatly enhanced in the presence of a non-sensitizing catalase inhibitor, 3-amino triazole (Lerman and Borkman, 1976; Lerman et al., 1976a,b; Kuck, 1976a,b). Due to inhibition of catalase, the enzyme capable of reducing hydrogen peroxide in mammalian tissue (see for detailed information, section 1.4), a lower-than-normal glutathione concentration in lens might occur (Kuck, 1976a,b). Since glutathione, which is present in the lens in relatively high concentration as is also ascorbic acid (Kuck, 1975), may act as a radical scavenger as well as a reductor of hydrogen peroxide (Slater, 1972), lens reactions which proceed via free radicals may be favored when glutathione or ascorbic acid concentrations decrease.

To summarize, natural short-wavelength ultraviolet light (<300 nm) is probably not an important factor in aging and nuclear cataract formation in the human lens. Near-UV-light (300-400 nm) penetrates the lens at young age very easily, but decreasingly with the development of lens pigmentation. Although photo-oxidation of tryptophan, the most near-UV-light susceptible amino acid in lens proteins, may result in yellowing, cross-linking and insolubilization of proteins, hard evidence is missing to support the hypothesis that destruction of tryptophan causes these effects in the human lens nucleus. The UV-hypothesis, postulating that sunlight causes nuclear cataract in old human lenses, seems to be transformed to a free radical hypothesis in which tryptophan

is involved in a non-destructive sense, generating free radicals and other oxidants via secondary processes, which may challenge the lens defense system throughout aging. Lack of free radical scavengers may cause the onset of nuclear cataract and may lead to the production of more free radicals and hydrogen peroxide. Eventually, yellowing of aged proteins occurs, but it should be stressed that aging of the lens, including age-related changes in proteins and plasma membranes, seems to determine when and where cataract is to develop.

More aspects of the human lens will be discussed in the following sections, in principle, only literature after 1975 has been considered, as preceding reports on aging and cataractogenesis of the lens have been surveyed by Harding and Dilley (1976).

1.1.3. Lens weight, water content, protein content and ion concentrations

The lens consists largely of protein, less than roughly 5% of the lens dry weight may represent non-protein material (Kuck, 1975). In human lens, 30-35% of the wet weight turned out to be protein (van Heyningen, 1972b; Klethi, 1976). The wet weight of human lenses (60-80 years of age) does not change significantly upon nuclear cataract development (Kramps et al., 1976; Kramps, 1977). The water content is relatively independent of age (Fisher and Pettet, 1973), no differences have been found between the water content of

cortices and nuclei from both normal and nuclear-cataractous lenses, although the latter lenses reveal nuclei of hard consistence (Kramps et al., 1976; Fisher and Pettet, 1973). The thickness of the lens capsule and its permeability to water change with age, but do not change upon nuclear cataract development (Fisher, 1978). The elasticity of the lens capsule decreases with age (Fisher, 1978). In nuclear-cataractous lenses, the total amount of proteins is not significantly different from that of normal lenses of the same age (Kramps, 1977). Syneresis, i.e. change in hydration of proteins, might occur in these lenses, as suggested by Bettelheim (1979) and Racz et al. (1979), causing changes in refractive index fluctuations and, with increasing size of light-scattering molecules, causing lens turbidity. The result of syneresis would not be a change in water content of lens parts, but a change in water distribution among the media surrounding the protein molecules and the hydration sphere of the proteins. The distribution pattern of water in bovine lens has been given by Rink (1978).

Normal values of sodium, potassium, chloride, magnesium and calcium concentrations and also normal protein-binding behavior of these ions have been found in nuclear-cataractous lenses, which findings are in contrast to the situation in other senile cataractous lenses (for review, cf. Bellows, 1975; Duncan and van Heyningen, 1976, 1977; Bushell and Duncan, 1978; Fein et al., 1979).

The protein concentration (the total concentration may

become 900 mg/ml) increases throughout the human lens (Bando et al., 1976). In calf lens, a discontinuous (van Kleef, 1975) and in rat lens (Philipson, 1969) a continuous pattern of protein concentration has been described. The human lens grows throughout life without shedding cells; lens thickness and age are linearily related. Synthesis of proteins cannot be found in the nuclear region of the human lens (Dilley and van Heyningen, 1976), which findings are in accordance with those of Wannemacher and Spector (1968) for calf lenses.

1.2. Changes in lens structural proteins upon aging and nuclear cataractogenesis

1.2.1. Introduction

With a life expectancy of more than 70 years, man is equipped with a lens in which the proteins remain fairly stable and reveal only a few changes. After homogenization of the lens, problems arise in keeping the lens proteins in the parent state (for review, cf. Harding and Dilley, 1976). Another problem, describing changes in lens structural proteins is that, at present, no generally adopted method exists for the isolation and separation of proteins. Therefore, the estimates of the protein contents and, additionally, the composition of protein fractions differ considerably.

In aging lens proteins degradation, aggregation, insolubilization and amino acid modification processes occur, which also continue to occur during nuclear cataract development. At any time one can take a snapshot of the human lens, but

by comparing normal with cataractous lenses, cortices (young material) with nuclei (old material), prenatal with juvenile and adult lenses, we try to understand what has happened in the lens.

In general, lens proteins are studied after homogenization of the lens in a buffer solution, varying in pH (6.8-8.6), volume, ionic strength, and containing variable amounts of preservatives, proteinase inhibitors, metal chelators, anti-oxidants and recently cytoskeleton stabilizers. The distribution of proteins in the individual human lens at the condition chosen, among the water-soluble, water-insoluble and high molecular weight (high-M_r) proteins depends on the centrifugation force applied. According to a combination of results, obtained by Zigman et al. (1976a), Roy and Spector (1976a) and Harding (1977), we shall call the protein fraction centrifuged down at 9,900 g, 25 min, 4°C: water-insoluble (WI), the supernatant can be centrifuged at 100,000 g, 55 min, 4°C, resulting in a pellet, which contains high-M_r proteins, and a supernatant, containing low molecular weight proteins (low-M_r) in a relative sense. The latter two fractions may also be isolated on a Bio-Gel A-5m column from the 9,900 g supernatant.

After removal of water-insoluble material, individual water-soluble proteins may be isolated by chromatographic, electrophoretic or centrifugal methods. Protein contents of fractions should be determined both by measuring the absorbance at 280 nm and by the Lowry method (Harding, 1977).

Chromatographic behavior of lens proteins may depend on the type of column matrix used, on temperature, protein concentration and protein pretreatment, which are factors still not fully taken into consideration (Harding and Dilley, 1976; Harding, 1977; Bindels et al., 1978; Li, 1979). The lowest molecular weight lens proteins seem to be the most susceptible to changes induced by temperature rising (Li, 1979).

The water-soluble proteins of the human lens are separated by means of gel exclusion chromatography into more than 6 fractions, viz. high-M_r, α (low-$M_r\alpha$), β_1, β_2, β_3, γ fraction and others. Gamma fractions and others may contain non-protein material like tRNA, non-protein-bound pigments etc. The proteins eluting have been named crystallins, with the prefix indicating their chromatographic elution order and, roughly, the type of protein.

The water-insoluble lens fraction contains aged proteins, plasma membranes and the cytoskeleton; its composition changes upon aging and nuclear cataract development. Methods for the isolation of water-insoluble proteins have been summarized by Roy and Spector (1978a). The use of detergents and high temperature effects during extraction of membrane proteins has been pointed out by Wong et al. (1979), Horwitz et al. (1979) and Broekhuyse and Kuhlmann (1979).

1.2.2. Loss of small polypeptides and protein degradation

When the water-soluble proteins from normal and nuclear-cataractous human lenses (60 to 80 years of age) are sepa-

rated by gel chromatography, a decrease in the proportion of the γ-fraction seems to occur upon nuclear cataract development, in particular in the nuclear region of the lens (Kramps et al., 1976; Kramps, 1977). Studying age-groups of human lenses, Lerman (1976) found a decrease in the proportion of the γ-fraction in the water-soluble proteins from 25% (10-19 years of age) to 9.7% (80-89 years of age). On aging in individual human lenses (22-82 years), a continuing decrease of polypeptides composing the γ-fraction has been found on gel chromatography (Jedziniak et al., 1978a) as well as on ion-exchange chromatography (Coghlan and Augusteyn, 1977) for lenses of 0 to 90 years of age. Kabasawa et al. (1977a; 1978) found an age-related change in the γ-fraction composition; in older human lenses, a high molecular weight γ-crystallin (γ_H) was found, occurring in age-dependently higher amounts in the cortex than in the nucleus. Sandberg and Closs (1978a, 1978b,c) have found γ-crystallins in β- and even in α-crystallins on ion-exchange chromatography; however, they found no decrease in γ-crystallin antigen concentration in the water-soluble γ-fraction from cataractous human lenses.

The human lens γ-crystallins (γ-fraction) consist of a series of monomeric proteins with molecular weights ranging from 27,000 to 10,000 daltons (Kramps, 1977; Ringens et al., 1978; Jedziniak et al., 1978a) and with isoelectric points ranging from pH 5.0 to 7.0 (Kramps, 1977; Ringens et al., 1978). On the basis of peptide mapping and amino acid analysis, Croft (1973) showed that most proteins composing the γ-

fraction from old and/or cataractous human lenses were similar to a protein, occurring in bovine lenses, known as β_S-crystallin. Kabasawa et al. (1977b), showed that in bovine lenses β_S- and γ-crystallins are immunochemically different proteins. Using immunochemical techniques, Liem-Thé (1975) revealed that the human γ-fraction contained proteins to be considered as γ-crystallins and others to be considered as β-crystallins.

The γ-crystallins can be distinguished from the majority of water-soluble lens proteins in that they show free NH_2-terminal amino acid residues (Björk, 1961).

Apart from the possibility that γ-crystallins are not present eventually in old and cataractous human lenses (Harding and Dilley, 1976; Papaconstantinou, 1967), it has been suggested that the decrease in the proportion of γ-fractions during cataract development might be due to:
a. leakage of proteins, composing the γ-fraction, out of the lens (François and Rabaey, 1958; Hockwin et al., 1973);
b. a change in solubility state of the γ-proteins either during extraction or by intrinsic factors like sulfhydryl oxidation (François and Rabaey, 1958; Hockwin et al., 1973) and c. association with or covalently cross-linking to higher molecular weight proteins (Harding, 1972a,b; Kramps, 1977; Ringens et al., 1978; Kabasawa et al., 1977a; 1978).

Compatible with the hypothesis that the γ-proteins leak out of the lens are the findings of Sandberg and Closs (1979); they demonstrated the presence of α- and γ-crystallin anti-

gens in the aqueous humor of the human eye. Nuclear-cataractous lenses revealed an increase in α- and a decrease in γ-antigen concentration.

Lens protein degradation has been studied in several mammalian lenses (Harding and Dilley, 1976; Bloemendal, 1977a,b). The existence of continual intracellular protein renewal and turnover, as found in various eukaryotic tissues (Schimke and Katunuma, 1975), seems not to hold for lens tissue. Protein degradation in the lens has been unequivocally found in α-crystallin, which is one of the major proteins in the mammalian lens. Recently, degradation of an intrinsic lens plasma membrane protein (26,000 dalton) has been found to occur in bovine lens (Bloemendal et al., 1977) after standing *in vitro* and in the human lens until the age of 50 years (Horwitz et al., 1979; Roy, 1979). In bovine lens β-crystallins, a post-translationally formed polypeptide has been postulated, which could be a degradation product of a newly-synthesized polypeptide; this presumed degradation product might be involved in the formation of a β-crystallin, viz. β_H (Vermorken et al., 1977).

Whether degradation occurs in β-crystallins on aging in human lens is not known, although Zigler (1978) also pointed to the possibility that it might occur in bovine lens.

Alpha-crystallin is the largest structural lens protein and has an weight-average molecular weight of about 850,000 daltons in bovine lens cortex. It is a spherical assembly of A- and B-type subunits, both of 20,000 daltons, occurring in

a 3:1 ratio (Siezen et al., 1978a; Siezen and Berger, 1978). A proposal for the quaternary structure of α-crystallin, consisting of three concentric layers of subunits, has been reported (Bindels et al., 1979). The model is based on experimental data obtained by electron microscopy (Siezen et al., 1978a), sedimentation analysis and small-angle X-ray scattering (Siezen and Berger, 1978), limited proteolysis (Siezen and Hoenders, 1977), bifunctional cross-linking (Siezen and Hoenders, 1979), dissociation/reassociation from urea (Siezen et al., 1978a; Siezen and Hoenders, 1979); sulfhydryl modification (Siezen et al., 1978b), partial dissociation in urea (Bindels et al., 1979) and investigation of effects on quaternary structure by pH, ionic strength, temperature and calcium concentration (Siezen et al., 1979; 1980). A consequence of all these studies is that the structure of the state, generally defined as "native" α-crystallin, is not stable under any of the experimental conditions, used in general to isolate lens proteins.

Although proteinases have been described to be present in bovine and human lenses (Harding and Dilley, 1976; Trayhurn and van Heyningen, 1976; Hahn et al., 1976; Hockwin et al., 1976; van Heyningen, 1978; Swanson et al., 1978), several arguments against their involvement in the selective COOH-terminal splitting of α-crystallin subunits have been raised (van Kleef, 1975). The activity of one protease, leucine aminopeptidase is highest in the inner region of the lens (van Kamp, 1974); it might be involved in the destruction of

proteins derived from cell organelles which disappear upon differentiation. It has been proposed (van Kleef et al., 1975; van Kleef, 1975) that the truncation of α-crystallin subunits is the result of non-enzymatic rupture of chemically labile bonds. Applying limited proteolysis *in vitro*, Siezen and Hoenders (1978) found that the α-crystallin B-type chains contain a site, which is susceptible to splitting, both *in vivo* and *in vitro*. For the A-type chains they proposed a model in which a part of the chain forms a loop, containing the *in vivo* cleavage site near the main *in vitro* cleavage sites. The presence of such a loop would be compatible with the finding that lens proteins are mainly in an anti-parallel beta-pleated configuration (Yu and East, 1975; Schachar and Solin, 1975).

Since the process of splitting and degradation is post-translational and occurs progressively in α-crystallin in the older parts of the bovine lens, it has been considered to be age-related (Stauffer et al., 1974; van Kleef, 1975; van Kleef et al., 1975; 1976; for review, cf. Bloemendal, 1977a,b) Since the splitting occurs very specifically, amino acid sequence- or conformation- dependent autolysis seems to have occurred to the aging α-crystallin (for review, cf. Robinson, 1979; McKerrow, 1979); in this process subunit structure, protein compactness, hydrophobicity, charge and size play a role (Siezen et al., 1980).

Larger subunits in proteins have a tendency to be degraded more rapidly than smaller ones (Schimke and Katunuma, 1975; Acton

and Gupta, 1979). Dice and Goldberg (1975) found a correlation between the logarithm of the subunit molecular weight and the half-life of several rat liver proteins. A parameter, relating the amino acid composition and the subunit size of a protein to the degradative rate *in vivo* has been described (Momany et al., 1976). A weak correlation exists between rates of degradation and isoelectric points (Acton and Gupta, 1979). Whether these findings can be applied to lens protein degradation remains to be elucidated.

Although γ-crystallins (27,000-10,000 daltons) have been reported to disappear during aging of the human lens, there is no report showing the presence of polypeptides of less than roughly 10,000 daltons in human lens. A very important fact may be that all structural lens proteins, but the γ-crystallins, have acetylated NH_2-terminal amino acid residues. Blow et al. (1975, 1977) argue that the only (metal-dependent) endopeptidase found in the lens, possesses most of the properties required for degradation of α-crystallin. Van Heyningen (1976) proposed that enzymatic proteolysis in lens occurs, when inactive complexes, containing the enzyme and α-crystallin, become loosened due to truncation of subunits. The enzyme is then activated and starts to degrade the loosened peptides, which are lost from the lens. In fact, COOH-terminal peptides from both degraded A- and B-type α-crystallin chains have never been found in lens homogenates (van Kamp, 1974; van Kleef, 1975). Interesting to note, however, is that Siezen and Hoenders (1978) could isolate

in vitro truncated α-crystallin chains, both the acetylated NH_2-terminal sequence as well as the COOH-terminal one; they used protease inhibitors to isolate α-crystallin intactly, omitting them during the experiments.

Involvement of calcium in the process of protease activation has been suggested, an increase in calcium concentration might be responsible for the low dry weight found for many cataractous lenses (van Heyningen, 1972a,b). As stated before, however, nuclear-cataractous lenses show no higher calcium levels than normal lenses of the same age.

In human lens α-crystallin, subunit degradation occurs too, as judged by several electrophoretic techniques (Kramps et al., 1976; Roy and Spector, 1976b,c; Kramps, 1977; Kramps et al., 1978a,b). Much less is known about the quaternary structure of human α-crystallin; it has been reported (Horwitz, 1976) that human α-crystallin shows secondary and tertiary structures similar to those of bovine α-crystallin. No differences in degradation rate were found in cortical and nuclear α-crystallin between normal and nuclear-cataractous lenses of 60 to 80 years of age (Kramps et al., 1976; Ringens et al., 1978; Roy and Spector, 1978c). Coghlan and Augusteyn (1977) found an age-dependent increase in water-soluble very acidic polypeptides, resembling α-crystallin in amino acid composition. Such polypeptides, occurring in old human lenses, may be identical with small polypeptides, which have been found in water-insoluble fractions (Roy and Spector, 1978b) and in all old human water-soluble fractions (Kramps et al.,

1978a,b; Kramps, 1977). These polypeptides may also be degradation products.

In old human lens nuclei, α-crystallin seems to be absent in the water-soluble fraction (Roy and Spector, 1976c). This finding was not confirmed by others (Kramps, 1977; Kramps et al., 1976; Kramps et al., 1978a,b; Ringens et al., 1978), who found in normal and cataractous lens parts the same ratio of high-M_r proteins over low-M_r α-crystallin in the water-soluble fraction. Roy and Spector (1976c) also suggested that the A-type chains of low-M_r α-crystallin are degraded at a much faster rate than the B-type chains, which fact could explain the accumulation of small A-type derived peptides in the water-insoluble fraction from old human lenses. A spontaneous degradation of egg albumin has been reported to occur (Saroff, 1977), which process could be due to an intrinsic enzyme-like activity associated with the sulfhydryl groups of this protein. Interesting to note is that the human A-type chains contain two cysteine residues (Kramps, 1977), whereas the bovine A-chain contains only one. B-type chains from bovine and human α-crystallin contain no cysteine residues (Kramps et al., 1977).

1.2.3. Light-scattering, protein aggregation and protein insolubilization

Applying light-scattering principles to the turbidity of human cataractous lenses, Benedek (1971) calculated that if 20% of the lens proteins were insoluble with a molecular

weight greater than $5x10^7$ daltons, the lens would be turbid. Using the technique of optical-mixing spectroscopy (protein diffusivity measurements utilizing laser light-scattering spectroscopy), Tanaka and Benedek (1975) demonstrated the presence of protein aggregates (about $5x10^8$ daltons) in intact cataractous human lenses, but none in normal lenses. Quantitatively, the existence of high molecular weight proteins in intact normal human lenses was verified by Jedziniak et al. (1978b), using quasi-elastic light-scattering. The concentration of high-M_r proteins increased monotonically with age, being absent in the infant lens and representing 3% of the total soluble lens protein at the age of 60 years. Since 1971, between 2 and 39% of the protein of transparent human lenses of people, aged 50-70 years, has been isolated as high-M_r proteins. More high-M_r proteins were isolated from the nuclear region of the lens, pointing to an age-dependent process of protein aggregation (Spector et al., 1974; Jedziniak et al., 1975), whereas others found no age-dependent changes in the high-M_r protein level (Dilley, 1975b). Since almost all crystallins seem to decrease in proportion on aging of the human lens (for review, cf. Harding and Dilley, 1976; Coghlan and Augusteyn, 1977; Jedziniak et al., 1978a), it is now well accepted and confirmed by biochemical evidence that, in contrast to calf and bovine lens, human lens high-M_r crystallins are not composed only of α-crystallin or its subunits.

During aging and, in particular, during nuclear cataract

development, the proportion of high-M_r proteins in the water-soluble fraction seems to increase, the border between the high-M_r proteins and the insoluble proteins becomes fluent and the definition of these fractions becomes operational and more or less arbitrary. More cataractous state-dependent aspects will be discussed in other sections.

Recently, Harding (1977) discussed the presence and quantitation of high-M_r aggregates in homogenates of the human lens. Widely different amounts have been isolated by different authors from apparently similar lenses (Harding and Dilley, 1976), conditions of centrifugation of the lens homogenate seem to be critical for the isolation of lens fractions or high-M_r proteins (Roy and Spector, 1976a). The presence of anti-oxidants, which are mostly of the sulfhydryl type, is also an important factor (Dilley, 1975b, Kramps et al., 1976, Kramps et al., 1978c). In addition, only 50% of the high-M_r fraction, isolated from the 9,900 g supernatant, turned out to be true protein (Harding, 1977).

The polypeptide chain composition of the high-M_r and water-insoluble fraction of human lenses led Roy and Spector (1976c, 1978a,c) to the proposal that the following precursor relationships may exist:

low-M_r → high-M_r → water-insoluble but urea-soluble → urea-insoluble proteins

Accordingly, high-M_r proteins are the precursors of insoluble aggregates, found in moderate amounts in normal aging lens (Garner and Spector, 1979) and strongly increasing amounts in

nuclear-cataractous lenses (Kramps et al., 1976; Takemoto and Azari, 1977). Insolubilization has been found chiefly and progressively in the nucleus of nuclear-cataractous lenses (Kramps et al., 1976; Kramps, 1977).

Various causes for the aggregation of lens proteins to insoluble proteins have been proposed; these include calcium, disulfide and non-disulfide covalent cross-links as reviewed by Harding and Dilley (1976).

The state of sulfhydryl groups in the proteins from the nucleus of 16 normal human lenses (18-63 years) was investigated by Anderson and Spector (1978). The water-insoluble proteins, which increased in proportion from 20% to 55% with age, showed a total level of sulfhydryl + disulfide groups ranging from 100-170 µmol/g protein and no trend of this level with aging. The water-soluble proteins, regardless of age, had a much higher level (200-290 µmol/g protein). Definite changes in the distribution of protein-SH and -SS- groups were found to accompany aging. In the 60-year-old lenses disulfides in the insoluble or soluble protein averaged higher than in the younger ones and amounted to 15-30% of the -SH + -SS- level. The total amount of nuclear -SH + -SS- groups turned out to remain reasonably constant. It could not be deduced from these experiments whether or not the -SH groups of soluble proteins are oxidized prior to or after they become insoluble. Under anaerobic conditions and in the absence of reducing agents, disulfide bonds may be present in the structural lens proteins (Takemoto and Azari, 1976), but

according to Kramps (1977, Kramps et al., 1978c), they are intramolecular.

As far as nuclear-cataractous lenses are concerned, only in the nucleus of this type of cataractous lenses, non-membrane proteins are present that do not dissolve in urea, guanidine chloride and/or dodecyl sulfate under anaerobic conditions and in the presence of reducing agents (for review, cf. Harding and Dilley, 1976). The dissolved proteins contain only intramolecular disulfide bonds, although bonds between proteins and small peptides or sulfhydryl group containing low molecular weight compounds (like glutathione) could not be denied (Kramps et al., 1978c, Kramps, 1977). In contrast, Roy and Spector (1978c) found in cataractous lens water-insoluble fraction a disulfide-linked high-M_r aggregate, composed of several polypeptides (after reduction).

Empirically, the high-M_r proteins, which do not dissociate in agents like urea, guanidine chloride and/or dodecyl sulfate in the presence of reducing agents, have been considered to contain non-disulfide covalent bonds (Harding and Dilley, 1976, Kramps et al., 1978c). Under the same conditions, lens proteins, which do not dissolve in dissociating agents in the presence of reducing agents have been considered to contain many non-disulfide covalent bonds (Takemoto and Azari, 1977, Harding and Dilley, 1976), since these proteins are colored, it might be expected that the non-disulfide covalent cross-links are identical with the colored compounds found in these protein fractions (Pirie, 1968, Buckingham, 1971, 1972). Non-

tryptophan fluorescence, which increases during the development of nuclear cataract, is also associated with these cross-linked protein fractions (Dilley and Pirie, 1974, Augusteyn, 1975).

Products with β-carboline structure (see Figure 1.2, upper line) were isolated from cataractous lenses by Dillon et al. (1976). These compounds were also found in 45- and 77-year-old normal human lenses, being absent in young human and calf lenses. Indole ethylamine and an aldehyde seem to be the precursors for the formation of the β-carboline structure (Barker et al., 1979), the missing link between the Pictet-Spengler reaction (equation 2) and the compounds found by Dillon et al. (1976) seems to be hydrogen peroxide, necessary to abstract hydrogen atoms (Hess and Udenfriend, 1959). Moreover, 3,3'-bityrosine (eq. 6) and anthranilic acid (o-amino benzoic acid) were found in cataractous lenses after proteolytic digestion of the yellow protein fraction (Truscott and Augusteyn, 1977a, anthranilic acid, Garcia-Castineiras et al., 1978a,b, both compounds). Bityrosine seems to be a peroxidation product of tyrosine (Gross and Sizer, 1959) and has been found in resilin (Andersen, 1964), elastin (LaBella et al., 1967), sea urchin egg fertilization membrane (Foerder and Shapiro, 1977) and mammalian collagen (LaBella et al., 1978), although not yet in old lens capsule. Anthranilic acid was absent from the digest of normal lenses and was suggested to be an oxidation product of tryptophan, it seems to be bound covalently to lens proteins and the precursor, from

which it may be derived, is susceptible to enzymatic hydrolysis rather specifically (Truscott and Augusteyn, 1977a). Lerman and Borkman (1978a) measured fluorescence lifetime in human lenses and compared the obtained value with that for anthranilic acid: almost identical lifetimes were found.

Whether the compounds, described before, serve as cross-links between lens structural proteins is still an open question; obtaining very small amounts of these compounds by enzymatic digestion or acid hydrolysis does not necessarily mean that they are present in small amounts in the parent material. Kramps (1977) failed to detect bityrosine in cataractous European lenses; he also searched for the presence of collagen-like cross-links and isopeptide bonds in nuclear cataracts but his results were negative.

1.2.4. Racemization and deamidation of protein residues

Recently, the racemization of aspartyl residues has been added to the list of age-related changes taking place in the proteins of the human lens (Masters et al., 1977; Helfman et al., 1977; Masters et al., 1978; Garner and Spector, 1978). Racemization of amino acids (in particular, aspartic acid) seems to be a measure of the aging of biological material (Williams and Smith, 1977).

In the inner region (15% of the wet weight) of the normal human lens, racemization takes place at a constant rate of 0.14% per year (Masters et al., 1977), without being accelerated upon nuclear cataract development (Masters et al., 1978).

Garner and Spector (1978) investigated the D/L-aspartic acid ratio of the water-insoluble (60,000 g pellets in their set-up) and water-soluble fractions (60,000 g supernatants) from normal and cataractous lenses. They found a D/L-Asp ratio of 0.036 for the water-soluble fraction of whole lenses, remaining quite constant with age. For the water-insoluble fraction (including the high-M_r proteins in this set-up) an increase in D-Asp content of 0.17% per year was found, which rate is comparable with the results obtained by Masters et al. (1978) for their water-insoluble fraction (25,000 g pellet, 0.18% per year). Upon cataract development, a decrease in the abundance of D-Asp was found in the water-insoluble fraction (Garner and Spector, 1978). It was concluded that this decrease might be due to the accumulation of aggregated and insolubilized proteins that were low in D/L-Asp ratio.

In the normal aging process, the racemization of aspartic acid may contribute to perturbation of lens protein structure, which may result in aggregation and insolubilization. The rapid insolubilization of proteins during nuclear cataract development is not due to increased racemization in water-soluble proteins. However, remarkable differences have been found between the D/L-Asp ratios of polypeptides isolated from the water-soluble and water-insoluble fraction of cataractous lenses (Garner and Spector, 1978, Masters et al., 1978). Most polypeptides (predominantly a 10,000 dalton protein), found in the water-insoluble fraction of cataractous lenses, revealed high D/L-Asp ratios.

It has become evident that de novo synthesized A_2 and B_2 polypeptide chains of bovine α-crystallin are subject to two types of post-translational modifications: deamidation (Schoenmakers and Bloemendal, 1968) and COOH-terminal chain shortening (see section 1.2.2., for the latter process). The deamidation products, A_1 and B_1, appear only in the later stages of foetal life and reach their highest level in old bovine lens cortex, where another putative deamidation product becomes prevalent (van Kleef et al., 1976). Deamidation occurs in α-crystallin of many vertebrate species (de Jong et al., 1976). It seems justified to consider degradation of α-crystallin as an aging process, whereas deamidation is not age-related (van Kleef et al., 1976; Harding and Dilley, 1976; Dilley and Harding, 1975). The polypeptides of α-crystallin from 60- to 80-year-old human lenses have been studied to determine whether they have been subject to similar post-synthetic alterations as known from bovine α-crystallin (Kramps et al., 1978a,b). The human and the bovine α-A_2 chains differ in 10 out of 173, the α-B_2 chains in 3 out of 175 positions (de Jong et al., 1975; Kramps et al., 1977). Other major polypeptides turned out to be deamidated A_2 of normal length (172 residues), shortened A_2-chain (A_2^{1-151}), shortened B_2-chain (B_2^{1-170}) and deamidated B_2 of normal length (175 residues). Additionally, a group of polypeptides with very low isoelectric points was found, which revealed a heterogeneous molecular weight below 18,000 dalton and a high degree of similarity with A-chains, as judged by tryptic peptide mapping,

these polypeptides were present in low proportion. The authors concluded that, in spite of several differences in subunit composition, comparable deamidation and degradation processes take place in human and bovine α-crystallin as a function of age.

It has been stated (Harding, 1976) that, since the changes described that occur in the lens appear to be post-translational, it is not relevant to bring up hypotheses ascribing age-dependent changes to transscriptional or translational events.

Acceleration of the rate of deamidation in some proteins and peptides has been reported to be caused by ascorbic acid or its oxidation products (Robinson et al., 1973).

1.3. Calcium, sugars, amino acid oxidation and protein aggregation

Investigators are still debating the role of calcium in human lens protein aggregation. It has been suggested that calcium may be required to induce aggregation, it seems to be not required to stabilize such macromolecules (Fein et al., 1979). Molecular alignment of lens proteins into a matrix by phosphopeptides has been suggested to occur on the basis of determination of X-ray diffraction patterns (Bettelheim and Wang, 1977) and calcium may be involved in the formation of this matrix. Nuclear-cataractous lenses revealed normal calcium levels (see section 1.1.3.), but it has been shown that in lenses with high calcium content no increase in

high-M_r aggregates occurs; on the contrary, a significant decrease in the amounts of these aggregates has been observed (Bushell and Duncan, 1978; for review, cf. Harding and Dilley, 1976). Calcium may play a role in the formation of subcapsular cataract due to fiber swelling (Fagerholm, 1979).

It has been suggested that glycosylation of lens crystallins plays a potential role in diabetic cataract formation (Stevens et al., 1978); non-enzymatic glycosylation of proteins might be an early stage in the Maillard reaction or "non-enzymatic browning" of proteins, that occurs with aging (Ellis, 1959; Reynolds, 1965). Reducing sugars can form adducts with amino groups; subsequent to Schiff-base formation, the aldimine or ketimine undergoes an Amadori or Heyns rearrangement to form stable products, including progressively formed disubstituted and possibly multisubstituted sugars (Anet, 1958). Also furfuraldehyde may be released, due to internal rearrangement, which can also react with amino groups (Anet, 1964). Depending on temperature, pH, type of amino acid and sugars, compounds are formed that are fluorescent and yellow to brown (Rice et al., 1947).

Sugar in the decapsulated lens is predominantly linked with proteins, in particular with the plasma membranes (for earlier literature, cf. Bellows, 1975; Kramps, 1977; Bando et al., 1978; Alao, 1977). No obvious changes in the sugar content of the human lens have been found upon development of nuclear cataract (Bellows, 1975; Kramps, 1977; Bando et al., 1978; Pfaffenberger et al., 1976; Pande et al., 1979).

In normal human lenses a slight increase in protein-bound sugar has been found upon aging in all lens fractions (Bando et al., 1978). Conformational changes in individual lens proteins due to glycosylation might lead to increased susceptibility of sulfhydryl groups to oxidation, which is a characteristic of cataract development (Harding and Dilley, 1976).

By far, myoinositol is the main free polyol in the human lens; only traces of glucose and glucitol have been found, whereas other free polyols and aldoses have not been identified yet (Pfaffenberger et al., 1976). Galactose, glucosamine, fucose, sialic acid, glucose, mannose and hexosamine have been reported to be present in lens proteins (for review, cf. Kuck, 1975).

Free and protein-bound amino acids in normal and nuclear-cataractous lenses have been studied by several investigators (for review, cf. Kuck, 1975; Dilley and Pirie, 1974; Zigler et al., 1976a,b; Truscott and Augusteyn, 1977c). The total concentration of free amino acids was reduced by greater than 50% in nuclear-cataractous lenses (62-89 years of age) relatively to that in normal lenses (42-62 years) (Zigler et al., 1976a). Each individual amino acid decreased by at least 25% during nuclear cataract development. Comparison of values of amino acids (after acid hydrolysis) from the soluble and insoluble lens fraction showed quite similar patterns. The only difference between the insoluble fractions from normal and nuclear-cataractous lenses was the amino acid cysteine; it was suggested that the increase found after hydrolysis of

cataractous fractions in the presence of reducing agent (thioglycolic acid) might be due to increased disulfide bond formation in nuclear-cataractous lens proteins. Traces of kynurenine and cysteine were found in the free amino acids pool, small but definite amounts of kynurenine were present in most lens hydrolyzates. It could not be deduced from these findings whether kynurenine was originally present as N_1-formyl-kynurenine in the parent material, it is known that the latter oxidation product of tryptophan is acid-labile.

Since several investigators found no decrease in tryptophan level in the proteins of nuclear-cataractous lenses (Dilley and Pirie, 1974, Zigler et al., 1976b), it was suggested that kynurenine found in lens protein hydrolyzates might be derived from the free amino acids (tryptophan, kynurenine, N_1-formyl-kynurenine) with subsequent binding to lens proteins. However, since both a definite decrease in free tryptophan level as well as in that of other amino acids has been found (Zigler et al., 1976a), the conclusion that photo-oxidation products of free tryptophan are involved in the process of lens protein yellowing upon nuclear cataract development is not warranted (Dilley, 1975a). N_1-formyl-kynurenine might be present in the human lens, since it is involved in a pathway, converting enzymatically tryptophan into hydroxy-kynurenine, which is then conjugated with glucose (van Heyningen, 1971, 1973a,b, 1975). The product, a 2-hydroxy-kynurenine-glucoside may absorb short-wavelength

light (300-400 nm) before it strikes the retina. The amount of this compound decreases with the development of nuclear cataract (van Heyningen, 1973a,b). The human and primate lens metabolizes tryptophan, therefore, whereas the lens of other species does not (van Heyningen, 1976).

More recently, Truscott and Augusteyn (1977b), described the presence of methionine sulfoxide in nuclear-cataractous lenses. Until 60% of protein-cysteine had been oxidized, no methionine sulfoxide was found. In advanced nuclear-cataractous lenses it was shown that, in proteins from the nucleus cysteine was present for 90% in oxidized form and 45% of the total methionine occurred as methionine sulfoxide. It was suggested that sulfur oxidation processes spread from the lens nucleus to the cortex during nuclear cataract development, probably resulting from simple oxidation of sulfur containing residues by oxygen or hydrogen peroxide (Truscott and Augusteyn, 1977b).

Anthranilic acid and bityrosine have been found in nuclear-cataractous colored protein (Truscott and Augusteyn, 1977a; Garcia-Castineiras et al., 1978b). Anthranilic acid has not been found in the free amino acid pool of the lens; moreover, by normal amino acid analysis it cannot be detected since this compound shows no reaction with ninhydrin. Bityrosine should be readily detected, if present, after amino acid analysis by measuring its fluorescence (Malanik and Ledvina, 1979).

Irradiating N_1-formyl-kynurenine, the precursor of the

kynurenine-glucosides, with sunlight or simulated sunlight at neutral pH, Pirie and Dilley (1974) found that it was converted to N-formyl-anthranilic acid and 4-hydroxy-quinoline. Although the indole ring was broken, these compounds could not be detected in irradiated solutions of Gly-Trp and Trp-Gly. It was argumented that quinoline could not very likely be formed within proteins. N-formyl-anthranilic acid, if derived from N_1-formyl-kynurenine within lens proteins, would be split out. Yields of N-formyl-anthranilic acid production from tryptophan were very low, as expected from observations by others (Asquith and Rivett, 1971), showing that the photoproducts of tryptophan account for less than 10% of the starting material.

1.4. Lens defense system against oxidative challenge

In view of the fact that the lens functions to refract light on the retina, it should be equipped with substances, which prevent damage to lens components due to light and free radicals. A putative defending role is played by kynurenine-glucosides (van Heyningen, 1973a, 1975), which also may prevent that damaging light reaches the retina.

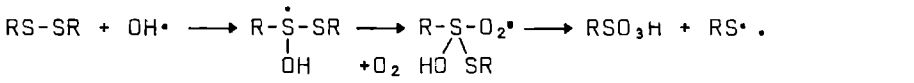
Glutathione (GSH) levels may be as high as 500 mg percent in the lens cortex of certain species (Kuck, 1975). In human eye bank lenses, 29 to 47 mg percent has been found. For many years, it was thought that GSH acted primarily as an *in situ* reducing agent, it might protect enzymes, having labile sulfhydryl groups in their active sites or it might maintain pro-

tein-sulfhydryl groups in the reduced state. It has been shown that GSH can cleave disulfide bonds in lens proteins, provided NADPH and glucose-6-phosphate is present (Augusteyn, 1979). A critical level of GSH in lens epithelium is required for maintaining normal cation transport (Giblin et al., 1976). Glutathione also rapidly converts dehydroascorbic acid to ascorbic acid, *in vitro*, by a non-enzymatic reaction (Kuck, 1975). Glutathione and ascorbic acid are considered to be potent radical scavengers (Slater, 1972). The lens has an active enzyme system for reducing GSSG (oxidized GSH), utilizing NADPH, produced by the hexosemonophosphate shunt. Both systems could serve to maintain the ascorbic acid level. The autoxidation of GSH within lens homogenates is very slow, no GSSG is found in the aqueous humor. Therefore, the decrease in GSH concentration in human lens during development of nuclear cataract (Truscott and Augusteyn, 1977d; Anderson and Spector, 1978) could be explained by assuming increased oxidation of sulfhydryl groups. Several analogs of GSH are present in the lens (for review, cf. Kuck, 1975); chief among these is ophthalmic acid (39 mg percent in calf lens), which does not contain a substituted cysteine residue like GSH, but, instead, α-amino butyric acid. It is difficult to figure how ophthalmic acid might act as a radical scavenger, but since it is also a competitive inhibitor of glyoxalase, it may be involved in maintaining normal levels of GSH, which might otherwise be withdrawn from the free pool, acting as a cofactor for this enzyme.

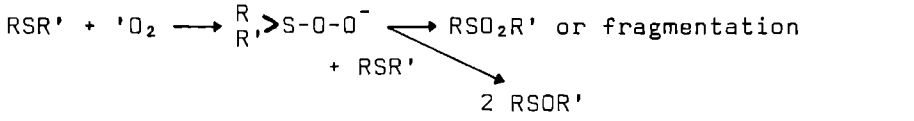
Ascorbic acid, a potent reducing agent, radical and singlet oxygen scavenger, but also a potent peroxidant via autoxidation (Bielski et al., 1975; Nishikimi, 1975; Bodannes and Chan, 1979; Sharma, 1977) is present in the lens in unusually high amounts in comparison with other tissues; the usual level is 20 to 30 mg/100 g lens tissue (Kuck, 1975). It has been difficult to find a relationship between ascorbic acid levels and nuclear cataractogenesis, its defending role in naphthalene cataracts has been proven (van Heyningen, 1976, 1978). Experimentally, ascorbic acid has been shown to accelerate deamidation of asparagine residues in peptides (Robinson et al., 1973). The lens cannot synthesize ascorbic acid, in the aqueous humor both dehydroascorbic acid (DHA) as well as ascorbic acid are found, the latter being asymmetrically distributed among the anterior and posterior chamber. According to Slater (1972), ascorbic acid (A) undergoes oxidation to dehydroascorbic acid (DHA) through the formation of monodehydroascorbic acid radical (MDHA$^\bullet$): A $\longrightarrow$ MDHA$^\bullet$ + H$^\bullet$ $\longrightarrow$ DHA + 2H$^\bullet$, acting thereby as a dual radical scavenger. Dehydroascorbic acid may be regenerated to ascorbic acid by GSH. Ascorbic acid radical (MDHA$^\bullet$) is a relatively non-reactive species, which decays mainly by disproportionation (Bielski et al., 1975).

Sulfur compounds, including GSH, may act as free radical reaction inhibitors (Slater, 1972; Nagata and Yamaguchi, 1978): RSH + R$^\bullet$ $\longrightarrow$ RS$^\bullet$ + RH, often terminating with dimerization to stable disulfide bonds, via RS$^\bullet$ + RS$^\bullet$ $\longrightarrow$ RS-SR. A mecha-

nism for radical scavenging by a disulfide bond has been discussed by Foye (1969):



Reactions between sulfides (like methionine) and singlet oxygen have been described by Monroe (1979):



Depending on the conditions, reactions between sulfides and oxygen or radicals may produce sulfoxides, sulfones and/or fragmentation products. Glutathione is not very effective in protecting animals against high doses of ionizing radiation, in which the production of very reactive radicals of the hydroxyl type overcomes the body's defense mechanism (Slater, 1972).

Besides GSH, ascorbic acid, thiols and sulfides, the lens has other protective mechanisms against oxidative damage, caused by oxidants generable *in vivo*, such as superoxide anions ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), lipid peroxides ($LOO^\bullet$) and lipid hydroperoxides (LOOH), singlet oxygen ($^1O_2$) and hydroxyl radicals ($OH^\bullet$). Various enzymes, present in the lens, may reduce these oxidants.

Superoxide dismutase (SOD), one of the main enzymes present in the lens, catalyzes the dismutation of superoxide and oxygen: $2\ H^+ + 2\ O_2^{-\bullet} \longrightarrow H_2O_2 + O_2$. Superoxide anions may be generated in the lens by autoxidation of, if present, flavins, quinones, thiols and epinephrine (Massey et al.,

1969; Fridovich, 1970; Misra and Fridovich, 1972a,b; Misra, 1974). Superoxide anions also can be destroyed by ascorbic acid and glutathione (Varma et al., 1977) at concentrations far below those found in normal and cataractous human lenses. Superoxide dismutase is present in lens epithelium, but not within the cortex or nucleus (Kuck, 1977; Varma et al., 1977; Bhuyan and Bhuyan, 1977b, 1978; Crouch et al., 1978). Superoxide anions and the dismutase product, hydrogen peroxide, can react, when traces of iron salts are present, to form hydroxyl radicals and singlet oxygen according to the Haber-Weiss scheme: $O_2^{-\bullet} + H_2O_2 \longrightarrow OH^\bullet + {}^1O_2 + OH^-$. Hydroxyl radicals and singlet oxygen are powerful oxidizing agents; reactions with cholesterol (Suwa et al., 1977) sulfides and amino acids (Monroe, 1979; Matheson and Lee, 1979; Kacher and Foote, 1979) have been described.

Hydrogen peroxide can be formed by the dismutation of superoxide anions, by irradiating tryptophan (McCormick et al., 1976), by autoxidation of ascorbic acid (Pirie, 1965) and by flavin-linked oxidase action on amino acids and glucose (Bright and Porter, 1970). It can be further reduced to water due to the catalatic action of the enzyme catalase: $H_2O_2 \rightarrow 2\ H_2O + O_2$. Catalase, however, plays a dual role; at high concentrations of hydrogen peroxide, the catalatic activity is predominant (see above), whereas at low concentration ($< 10^{-6}$M) and in the presence of a suitable hydrogen donor, e.g. L-dopamine (Awasthi et al., 1975) or formate (Chance and Herbert, 1950), catalase displays its peroxidative activity; it may, then, re-

duce hydroperoxides, in general, to water and alcohols: $ROOH + XH_2 \longrightarrow H_2O + ROH + X$, wherein XH_2 is the hydrogen donor. Catalase has been shown to be present in lens capsule, epithelium and endothelium, whereas it is almost undetectable in lens fibers (Bhuyan and Bhuyan, 1970; 1975; 1977a). Both activities of catalase may be inhibited by 3-amino triazole (see section 1.1.2), a toxic and carcinogenic substance. Hydrogen peroxide, which is a substrate for catalase and an inhibitor of superoxide dismutase may induce oxidation of other cell components. Lenses incubated with hydrogen peroxide showed a decreased level of GSH, but lens ascorbic acid was unaffected (Fukui, 1976).

Hydrogen peroxide and lipid peroxides may be reduced by a seleno-enzyme, glutathione peroxidase, simultaneously oxidizing GSH to GSSG (Holmberg, 1968). Glutathione peroxidase is present in the lens capsule and epithelium (Bhuyan and Bhuyan, 1977a), lens cortex (Pirie, 1965), corneal epithelium (Anderson and Spector, 1971) and endothelium (Bhuyan and Bhuyan, 1977a).

Glutathione reductase is involved in maintaining normal levels of GSH and, indirectly, of ascorbic acid. This enzyme, which is present predominantly in the cortex of the lens (Rogers and Augusteyn, 1978), reduces GSSG to GSH, using NADPH. In nuclear-cataractous lenses, glutathione reductase activities were normal (Rogers and Augusteyn, 1978).

Lipid peroxides are produced by the abstraction of hydrogen (by a free radical) from unsaturated lipids and subse-

quent attack of oxygen (for review, cf. Slater, 1972; Tappel, 1973; Feeney and Berman, 1976; Fridovich, 1978). The formed lipid peroxide radical (LOO•) may capture a hydrogen atom from another unsaturated fatty acid molecule, thus triggering an autocatalytic chain reaction of lipid peroxidation, which can be terminated by reaction of two radicals together. Otherwise, in conjugated unsaturated lipids, a terminating step may be the splitting out of malondialdehyde (MDA); this compound is capable to form Schiff-bases, thus inactivating enzymes and cross-linking proteins. Malondialdehyde may form fluorescent compounds with amino acids (Chio and Tappel, 1969a,b). It is presumably the compound involved in the production of lipofuscin, age-pigment and other highly colored, fluorescent and cell damaging material, known to occur in old tissues of the human body (Strehler, 1964; Reichel, 1968; Porta and Hartroft, 1969; Shimasaki et al., 1977). Peroxidation products of lipids, like MDA, could not be detected in nuclear-cataractous human lenses (Kurzel et al., 1973). Peroxidizing lipids may react with amino acids and proteins (Schaich and Karel, 1975). Lipid hydroperoxides have been shown to react with cysteine in the presence of metal ions (Gardner et al., 1977).

1.5. Changes in lens plasma membranes upon aging and nuclear cataractogenesis

All biological membranes, including mammalian plasma membranes, are structurally based on a lipid bilayer (for re-

view, cf. Bretscher and Raff, 1975). Evidence for this concept comes from the susceptibility of the membranes to fracture across their mid-planes at low temperature. Into the lipid matrix, which provides the basic structure to the membrane, specific proteins are inserted asymmetrically and these are responsible for most of the membrane's functional properties. In the case of the plasma membranes, these functions include the receiving and transducing of various extracellular chemical signals, cell junction formation, transport of small molecules, endocytosis and exocytosis. Some of the membrane proteins span the bilayer; most of these, if not all, are glycosylated at the cytoplasmic side. Most of the polypeptides exposed on the cell surface, which do not span the bilayer, are likely held in place by association with other polypeptides which do so.

There are obvious advantages in having a fluid membrane, in which molecules can diffuse freely; it might facilitate cell locomotion and allow adaptable fits between cells. In the nucleus of nuclear-cataractous human lenses, strong deformation and degeneration of membranes has been shown to occur by electron microscopy (Kobayashi and Suzuki, 1975). In an aging normal lens, frequent formation of lacerated spaces occurs, due to destruction and liquefaction of some fibers. Globular bodies appear within the fiber cytoplasm, originating probably from disrupted, segregated and denaturated material from adjacent fiber cells. The fibers of senile cataractous lenses are much more irregular in shape and

width than those form normal lenses, interdigitations become more irregular and destruction, swelling and liquefaction of cells occurs.

Until recently, lens membranes were identically described as part of the so-called "albuminoid fraction", which remains when the major part of the lenticular mass has been dissolved in buffer solution. A portrait of plasma membrane specializations in eye lens has been given by Benedetti et al. (1976) and by Broekhuyse and Kuhlmann (1978). The small weight fraction of phospholipids must play an extraordinary important role in maintaining the integrity of lens cells. The lens synthesizes its own lipids and its independent of cholesterol in blood. The localization of lipids in the lens has been studied by Broekhuyse (1974). The predominant lipids in the mammalian lens are cholesterol and phospholipids, while in the human lens glycolipids are present in considerable amounts. The concentration of cholesterol in lens increases with age (Feldman, 1968); the increase can be attributed to a greater amount of cholesterol in the membranes of older fibers. The increase in sphingomyelin concentration upon aging of the human lens is most striking, a fourfold increase has been observed going from 3 to 80 years of age (Broekhuyse, 1974). Cholesterol and sphingomyelins constitute 72% of the phospholipids, while phosphatidylcholine has disappeared. This change in proportion has important implications on the fluidity of the old fiber membranes, since sphingomyelins may form compact aggregates with choles-

terol and stabilize the membranes. In the old lens nucleus plasma membranes show an exceptional high molar ratio of cholesterol over phospholipids of 2:1. Phospholipid metabolism in human lens is most active in epithelium and equator (Broekhuyse and Veerkamp, 1968; Broekhuyse, 1969).

In the plasma membranes of senile cataractous human lenses, the amount of proteolipid-protein extracted by polar solvent was less than for normal lens (Broekhuyse, 1969); this was interpreted as an indication for disintegration of membranes. It has been suggested that the disintegration would be secondary to the cataractous process (Broekhuyse, 1974). Lens plasma membranes seem to bind lens proteins, e.g. crystallins (Bracchi et al., 1971). Urea-treatment of isolated fiber membranes results in a dissociation of membrane-bound cytoplasmic "matrix" which consists mainly of crystallins (Broekhuyse et al., 1976; Broekhuyse and Kuhlmann, 1978). However, also extrinsic membrane proteins are stripped from the membranes by concentrated urea solution. The protein content of the urea-treated membranes is stable (Broekhuyse and Kuhlmann, 1978), while the lipid composition does not change during the isolation procedure (Broekhuyse and Kuhlmann, 1974). Quintuple layered membranes, seen by electron microscopy (Lasser and Balasz, 1972; Broekhuyse et al., 1976), can be concentrated by density gradient centrifugation of the membranes dissolved in deoxycholate (Benedetti et al., 1976). The main intrinsic membrane protein (M_r 26,500 daltons) from bovine lens has been isolated (Broekhuyse et al., 1976).

The cytoplasmic "matrix" is apparently bound to the lens plasma membranes (Broekhuyse and Kuhlmann, 1978). Aged cow lens α-crystallin has been shown to bind rather strongly to urea-washed membranes (Bracchi et al., 1971) and although others (Broekhuyse and Kuhlmann, 1978) could not reproduce these findings, it may be suggested that some of the aging crystallins may be considered as extrinsic membrane proteins. Recently, it was shown that a *de novo* synthesized α-crystallin subunit (A_2) associated with plasma membrane structures (Vermorken et al., 1979; Ramaekers et al., 1980). Moreover, α-crystallin has been shown to be closely attached to the cytoskeleton of the lens fiber, chiefly to the actin filaments (Kibbelaar, 1979). It has been suggested that the matrix of crystallins is bound to the membranes via the main intrinsic membrane protein (Broekhuyse and Kuhlmann, 1978) and is structured by microfilaments (Maisel and Perry, 1972; Maisel et al., 1976a,b; Benedetti et al., 1976; Broekhuyse et al., 1976). The role of cytoplasmic filaments in cell motility and cell shape has been reviewed by Gipson (1977) and Rafferty and Goossens (1978). A hypothetical structure of calf lens membranes has been given by Broekhuyse and Kuhlmann (1978).

The nature of the urea-insoluble material of the human lens has been described by Maisel (1977); it is composed of plasma membranes as well as some granular material. Enriched human lens plasma membranes have been studied more recently; the use of dodecyl sulfate and high temperature to investigate

membrane proteins should be avoided, as pointed to by several investigators (Wong et al., 1978; Horwitz et al., 1979; Broekhuyse and Kuhlmann, 1979). In human lens a main intrinsic membrane protein (M_r 26,000 daltons) has been described, besides another polypeptide of 22,000-23,000 daltons, which seems to be a post-translational degradation product of the first protein (Horwitz et al., 1979; Roy, 1979); the latter accumulates with age, mainly in the nuclear region of the lens (Horwitz et al., 1979). Degradation of the main intrinsic membrane protein in bovine lens has been described by Bloemendal et al. (1977). Recently, an extrinsic membrane protein (M_r 43,000 daltons) has been isolated (Spector et al., 1979), which is associated with high-M_r proteins in cataractous lenses. This protein has been found in the disulfide-rich fraction of water-insoluble proteins from cataractous lenses. It has been suggested that the association of soluble lens proteins and these membrane-associated polypeptides may lead to the formation of large aggregates, which may cause cataract. The 43,000 D polypeptide, however, also has a water-soluble and immunochemically identical counterpart. A diagram of the plasma membrane structure of the human lens fibers has been given by Spector et al. (1979). Binding of the 43,000 D protein to the membranes seems to be dependent on calcium and is stronger in younger lenses (Garner and Spector, 1979). In cataractous lenses, the 43,000 D polypeptide may have formed a disulfide-linked complex with putative crystallin polypeptides (Spector et al., 1979),

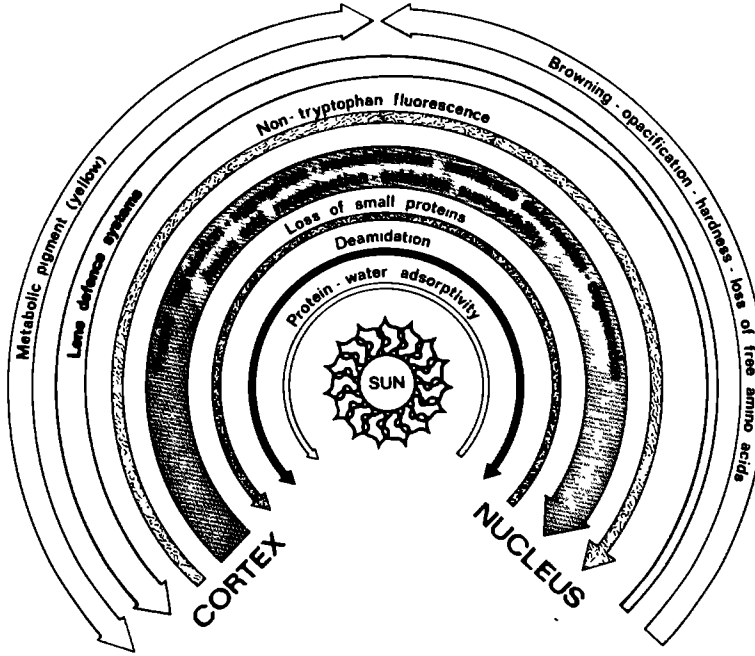
other polypeptides such as a 9,600 D and higher molecular weight species, that can be isolated from the water-insoluble fraction of cataractous lenses (Roy and Spector, 1978a,b,c; Roy et al., 1979), were found in this complex.

1.6. Summary and a scheme

Although at first sight the lens reveals only few changes upon aging, it has become evident that the order in which these changes occur and what induces them is still an open question. Many more problems arise in describing what happens in nuclear cataractogenesis. It is a fact that nuclear cataract develops when aging processes have proceeded relatively far, roughly over 40 years. If we suppose that sunlight might be involved in the development of nuclear cataract it is time to query the state of normal lenses at old age. Normal aging processes, like those described in the preceding sections, should then determine when and where nuclear cataract is to develop. In fact, aging processes continue during nuclear cataract development, but increased oxidation of protein residues and non-protein compounds seems to change the age-related distribution of lens proteins among aggregated water-soluble and water-insoluble fractions. The deformation of membranes, as seen in normal lens nuclei, is increased during nuclear cataract development. It cannot be deduced from literature at which solubility state oxidation of lens proteins takes place or whether deformation of membrane structure plays an "active" role in changing the solu-

bility state of lens crystallins. Co-operative effects may be involved in which racemization, degradation and deamidation within proteins is followed by aggregation and insolubilization due to increased oxidation susceptibility; on the other hand, changes in surface hydrophobicity of proteins due to aggregation may create interaction zones with membranes,

Fig. 1.3. Schematic representation of lens aging and nuclear cataract development



Following the direction of the arrow, increases in concentration and / or occurence of events have been found or postulated. Broadening arrows indicate discontinuity in the process meant. Double arrows indicate the obvious absence of age-related changes.

stabilized by covalent bonds formed as a result of oxidational stress. If we assume association between water-soluble proteins and a micro-filamentous matrix within the lens fiber cell, oxidation and aggregation processes may continue along this matrix, forming a rigid complex of oxidized, denatured material held together by the cytoskeleton until the burden is too heavy, membrane disruption as a result of continual stress on the anchoring proteins of the cytoskeleton, embedded in the plasma membranes, may then occur.

We have summarized all principal findings and postulates with respect to aging and nuclear cataractogenesis in Figure 1.3, which certainly is far from complete.

1.7. Aims of this study; scheme of approach

The major purpose of this study is to extend our understanding of any relationship between post-synthetical changes in structural lens proteins and lens fiber membranes and the process of nuclear browning occurring in old human lenses.

The preceding introduction shows that, as a result of the application of techniques from various disciplines the processes taking place in the lens during aging and nuclear cataractogenesis are becoming more understandable. However, on the molecular level the information is still far from complete, since the question remains why only the nuclear part of the human lens becomes hard, colored and its proteins hard to investigate, due to insolubilization. The processes of

deamidation, degradation and racemization have been proven to occur, but no experiments have been performed to elucidate why and how they occur so specifically in some of the lens proteins. The link between sunlight and the formation of nuclear cataract has been suggested to exist, but evidence is still missing to explain how energy from the sun may challenge and damage the lens components oxidatively, if not through the destruction of photo-labile amino acids and other components. If tryptophan is not the factor causing browning of proteins in a destructive sense, one may ask which components then are and whether there is another common factor involved, besides UV-light, in the production of lens nuclear color, fluorescence, membrane degeneration and protein cross-linking and insolubilization.

Several approaches that may shed more light on these events, have been followed in this thesis. Chemical determination of oxidation-susceptible lens constituents, enzymatic hydrolysis and analysis of cross-linked proteins and determination of *in vitro* peroxidation susceptibility of lens fractions may form the basis for detecting and understanding the pathway according to which nuclear cataract may develop in old human lenses.

There is strong evidence that UV-light causes nuclear cataract but autoxidation of lens components might do also. Reactive intermediates in the photo-oxidation process, if occurring, may induce peroxidative changes to lens components if they were not protected enzymatically or by other compounds,

designed by nature to trap reactive compounds. The pathway according to which nuclear cataract might develop and according to which we investigated it, is depicted in Figure 1.4.

As a starting point (chapter II) we investigated how the colored proteins from the human lens could be isolated and which relationship exists between the color of the lens nucleus and the spectral characteristics of lens fractions. In order to isolate lens parts, representing material which is synthesized at different ages, we performed experiments on the rheology of the lens.

In chapter III we report on the yellow protein fraction using methods developed in chapter II. By means of enzymatic digestion we isolated colored and fluorescent compounds which were further analyzed by several techniques.

The technique of pyrolysis mass spectrometry (chapter IV) permitted us for the first time to analyze almost parent lens material without troublesome isolation of compounds. The influence of the sun on the production of fluorescence in the human lens was investigated by comparing the amounts of the fluorescent compound, anthranilic acid, derived from European and Pakistani lenses. The pathway according to which this compound might arise and become associated with lens proteins was also investigated.

Chapter V and chapter VI describe the results obtained with modification studies employing sulfhydryl and tryptophanyl group reagents. These results might lead to the suggestion that non-protein-bound sulfhydryl group-containing

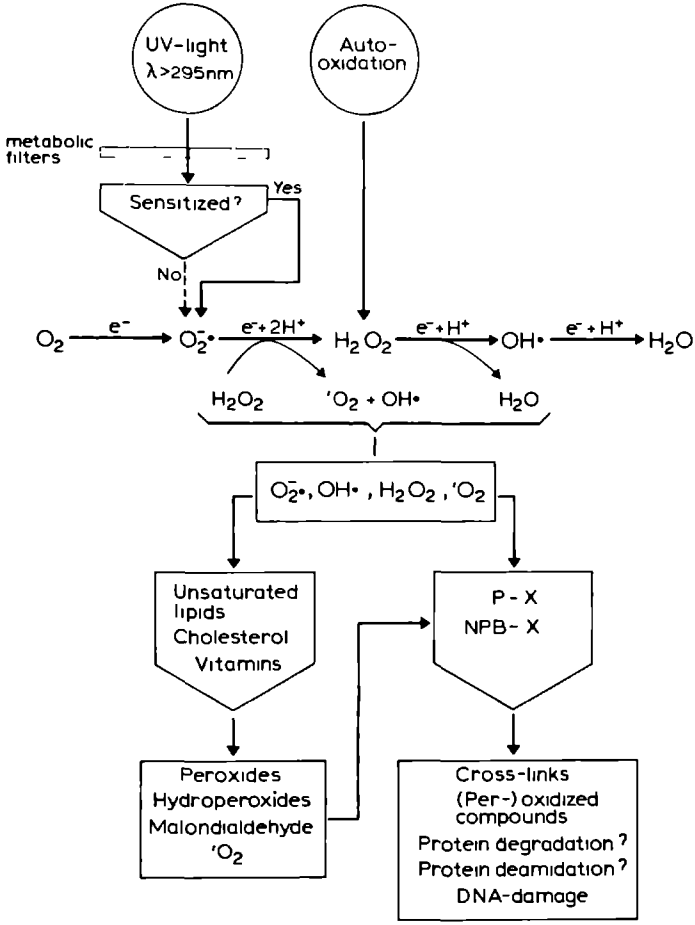
compounds are necessary in maintaining normal levels of disulfide bonds in proteins besides from defending the lens protein synthesis system against oxidative challenge. The determination of tryptophanyl levels may shed light on the question whether these residues are involved in the process of nuclear browning.

Chapter VII describes the results obtained by application of pyrolysis mass spectrometry to the oldest lens part, the embryonic nucleus, which may be the primary site for the development of nuclear cataract. It is a method which can be used to find out relationships between biomedical data like color of the lens nucleus as described by the ophthalmologist and the proportion of any component present in the total lens material or a part of it.

Chapter VIII describes the experiments in which we tried to find out whether peroxidation of lens membranes might have led to the formation of nuclear cataract in a broad sense.

Data concerning the primary structure of very acidic polypeptide chains were gathered (chapter IX) in order to establish whether they are involved in the post-synthetic alterations known to occur in aging lens tissue.

Fig. 1.4. Tentative pathway for the development of nuclear cataract in human lenses



For explanation of compounds see section 1.4
P-X, protein-bound components; NPB-X, non-protein-bound compounds

INTRODUCTORY EXPERIMENTS ON HUMAN NUCLEAR-CATARACTOUS LENSES

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2. INTRODUCTORY EXPERIMENTS ON HUMAN NUCLEAR-CATARACTOUS LENSES

2.1. Introduction

Human nuclear-cataractous lenses constitute a small fraction of the total number of senile cataracts observed clinically in temperate areas. Still, owing to their spectral characteristics and typical cataract location, they are favorites for investigation. A classification scheme, to be shown in this chapter and dividing nuclear-cataractous lenses into subgroups of increasing nuclear color, was found to be reflected by biochemical findings like increasing protein aggregation, cross-linking and insolubilization. It may be argued that such a classification scheme is rather subjective and, therefore, we investigated whether it could be refined or strengthened by spectral analysis of lens fractions, lens parts and non-protein-bound compounds. In order to investigate lens parts, to be separated on the basis of cataract location, we performed some experiments on the rheology of the lens. Based on literature findings and experiments to be described in this chapter, we designed an isolation pathway for individual lens proteins, in particular for the colored (yellow) protein fraction. Preliminary experiments on the yellow protein fraction will be described in this chapter.

2.2. *MATERIALS AND METHODS*

2.2.1. *Lenses and lens classification*

Before investigation of protein-chemical aspects of senile cataractous lenses takes place, lenses are classified, *in vivo* and *in vitro*, at the Eye Clinic of the Free University of Berlin by means of slitlamp biomicroscopy. Based on a combination of cataract location and intensity of opacity, color and age, the following classification scheme has been designed:

Patient's data: sex, age at the day of operation, special comments

Classification of the lens:

A Hypermature cataract: Morgagni
Shriveled
Intumescent

B Capsular cataract : Anterior
Posterior
Anterior-posterior (mixed)

C Cortical cataract : Anterior
Posterior
Anterior-posterior

Intensity of opacification: +, ++, +++
Water-clefts: +/-

D Nuclear cataract : Color: yellow
brown
dark brown
black (nigra)

Intensity of opacification: +, ++

For detailed information about types of cataract A, B, C, the reader is referred to Bellows (1975).

Most nuclear cataracts are accompanied with capsular cataract; investigating 38 lenses, sampled at random, 74% showed capsular cataract (32% being mixed capsular cataracts) and only 26% were pure nuclear-cataractous.

Considering these findings and the percentage participation of nuclear cataracts in temperate areas (estimated, 52°N.L.) as described by Pau (1976), only about 1.5% of all senile cataracts observed in The Netherlands and North Germany are pure nuclear-cataractous. Unfortunately, the occupation of patients (indoor-outdoor) was not tabulated; therefore the incidence of nuclear cataract in relation to sunlight exposition cannot be deduced from our lens classification files.

Lenses. Human nuclear-cataractous lenses were obtained from operations at the Eye Clinic of the Free University of Berlin (FRG). The lenses were classified *in vivo* by means of a slitlamp into four groups of increasing nuclear color: lenses with a yellow nucleus (Ye), a brown nucleus (Br), a dark brown nucleus (Db) and a black nucleus, ophthalmologically termed: cataracta nigra (Ni). Immediately after surgery and reexamination, lenses were stored in capped vials at -70°C. Transport to Nijmegen took place in a container filled with liquid nitrogen. To prevent lyophilization during storage (-80°C), lenses in capped vials were sealed in plastic tubings.

Normal, completely transparent lenses were obtained within 5 h after death of individuals, at various ages, from the Eye Hospital at Rotterdam, The Netherlands.

2.2.2. Rheological measurements

Rheological characteristics of human and calf lenses were determined by means of a Weissenberg Rheogoniometer model R 18. The concentric cylinder configuration was used for protein solutions and calf lens cortices, whereas the parallel-plate configuration was used for human lenses. Compression measurements were performed using an Instron Universal Testing Instrument.

Water-soluble proteins from calf lens cortices were isolated according to van Kleef and Hoenders (1973). A solution of lens proteins was prepared (30%, w/w) in 0.9% NaCl, pH 7.0. Normal and nuclear-cataractous lenses were slowly thawed, decapsulated, if necessary, and used immediately.

Visco-elastic properties of the lens were determined, applying very small deformations, in terms of the storage modulus G' and the loss modulus G''. The storage modulus G' defines the solid (elastic) character, the loss modulus G'' defines the liquid (viscous) character of visco-elastic materials. For an ideal elastic substance, G''=0, whereas for an ideal viscous substance, G'=0.

Compression data were handled by plotting stress T (=force/ $\frac{\Pi}{4}$ Wo^2; Pa) versus $\lambda^{-2}-\lambda$, in which W_o represents the equatorial width of the lens before compression and λ represents the ratio: lens axional height after over before compression. Force on the lens was applied via a crosshead from above and pressure measured from below.

2.2.3. Preparation of lens parts

Lenses were slowly thawed on an ice-cooled glass plate covered with parafilm to prevent slipping. After incision of the capsule at the anterior side, the transparent cortex (faintly yellow in color) was removed with a spatula, immobilizing the hard nucleus by means of a needle. This procedure took no more than 3 min per lens. Normal lenses were treated the same way, considering the inner soft 40-50% (wet weight) of the lens to be the nucleus. The nucleus was further subdivided into embryonic nucleus and surrounding hull, after trephinizing the nucleus with a cork borer (inner diameter, 4.5 mm), the embryonic nucleus cracked out itself.

2.2.4. Preparation of water-insoluble lens fractions

After decapsulation of the lenses, lens material was homogenized at 4°C in a pH 7.3 buffer (20 mM Tris-HCl), containing 1 mM EDTA and freshly prepared 10 mM DTE, in order to prevent proteolysis and sulfhydryl oxidation, respectively. About 10 ml per lens were used. Water-insoluble lens fractions were obtained by centrifugation of the homogenates at 9,900 g, 25 min, 4°C in a Sorvall-RC2B centrifuge using an SM-24 rotor.

2.2.5. Preparation of high-M_r lens proteins

Since the amounts of high-M_r proteins recovered from human lenses may depend on the centrifugation force applied, we investigated this, using the homogenate of 5 pooled normal human lenses (50 to 80 years of age), prepared as described

above. The homogenate was centrifuged, consecutively, at variable forces at 4°C (9,900 g, 26,000 g and 100,000 g, during 25,55 and 55 min, respectively), as described in literature (Roy and Spector, 1976a; Kramps et al., 1976; Zigman et al., 1977). Centrifugation at 9,900 and 26,000 g was performed in a Sorvall-RC2B centrifuge using an SM-24 rotor and at 100,000 g in a Spinco-L50 centrifuge using a Ti-50 rotor. Samples (2 ml each), taken from the consecutive supernatants, were applied to an agarose Bio-Gel A-5m column (exclusion limit 5×10^6 daltons; 2.5x100 cm). Fractionation was performed in homogenization buffer at a flow rate of 0.32 ml/min, maintained constant by means of a Cenco pump. The absorbance of the eluting fractions was measured at 280 nm using a Uvicord III absorptiometer (LKB, Sweden). The first two peak fractions, containing high-M_r proteins and low-M_r α-crystallin, were analyzed according to the Lowry method (Lowry et al., 1951), using bovine serum albumin as a standard (0-200 µg).

High-M_r proteins were also isolated as a pellet from normal and nuclear-cataractous lenses (10 lenses of each cataractous state) after centrifugation of the 9.900 g, 25 min supernatants again at 100,000 g, 55 min, 4°C.

2.2.6. Preparation of non-disulfide covalently cross-linked urea-soluble proteins

Individual nuclear-cataractous human lenses were decapsulated and homogenized in 10 ml pH 7.3 buffer (20 mM Tris-HCl,

80 mM NaCl, 1 mM EDTA), containing 7 M urea and freshly prepared 10 mM DTE. Stock urea solution (8 M) was kept in contact with mixed-bed ion-exchanger at 4°C, in order to avoid isocyanate formation. Homogenization buffer was prepared by diluting a more concentrated buffer solution with 8 M urea until the desired concentrations were obtained. After centrifugation (3,000 g, 15 min, 4°C) to remove insoluble material, 2 ml samples were taken from the supernatants and applied to an agarose Bio-Gel A-5m column (exclusion limit in urea 1×10^5 daltons, 1.6x70 cm). Elution was carried out at room temperature in urea buffer containing DTE at a flow rate of 0.15 ml/min. Fractions of 3 ml (75 drops) were collected.

2.2.7. Isolation of yellow protein fractions from nuclear-cataractous lenses

Four decapsulated normal and brown nuclear-cataractous lenses were homogenized in pH 7.3 buffer (20 mM Tris-HCl, 80 mM NaCl, 1 mM EDTA), containing 10 mM DTE. The homogenates were centrifuged (9,900 g, 25 min, 4°C) and the pellets re-extracted eight times. The water-insoluble fractions (pellets) were homogenized in pH 7.3 buffer, including 7 M urea. About 10 ml urea/buffer per 100 mg wet material were used. To the suspensions 0.1 ml 2-mercaptoethanol was added in order to reduce disulfide bonds. After 1 h, 0.2 ml ethylenimine was added. The aminoethylation of sulfhydryl groups was stopped by addition of 1 ml 2-mercaptoethanol. The reaction mixtures were dialyzed against water and lyophilized. The material ob-

tained from nuclear-cataractous lenses was pale yellow. This material is believed to contain membraneous material (Maisel, 1977) and, therefore, we extracted the lipids from the lyophilizates according to a method described by Cham and Knowles (1976), using a mixture of di-isopropylether: n-butanol: water (6:2:2, v/v); this solvent system has been used to delipidate plasma or serum without protein precipitation. Lyophilizates were first homogenized in water; a mixture of di-isopropyl-ether-n-butanol was then slowly added under agitation, until the desired volume ratios were obtained. The extraction procedure was carried out at $4^{o}C$ at 2 rpm during 16 h in stoppered glass tubes. After centrifugation (3,000 g, 15 min) a pellet, still yellow in color, was isolated from cataractous lenses only. The supernatant consisted of a water-layer, a fluffy coating and the organic solvent layer. The pellet was extracted again with di-isopropylether: water (8:2, v/v) to remove any butanol left. After centrifugation (3,000 g, 15 min) the yellow pellet was isolated and lyophilized. Afterwards, the material was repeatedly extracted with the pH 7.3 buffer, containing 7 M urea and 10 mM DTE. After centrifugation (3,000 g, 15 min) and repeated washings with water the isolation of the urea-DTE-insoluble delipidated protein was completed. The last pellet was lyophilized. It was not possible to estimate the molecular weight of this material because it did not enter any polyacrylamide gel (3-30% acrylamide). In one experiment, the yellow pellet, obtained by methods described above, was treated with sodium borohydride

to stabilize the cross-link, assuming that it contained an iminopropene structure. The yellow pellet was suspended in 0.05 M phosphate buffer, pH 7.8, and solid sodium borohydride (Fluka, Buchs, Switzerland) was added. Reaction was continued during 16 h at room temperature. After centrifugation (3,000 g, 15 min) the white pellet was repeatedly washed with distilled water to remove buffer salts and unreacted borohydride. The pellet was lyophilized and treated in the same way as the non-reduced yellow pellet.

2.2.8. Solubility of lens fractions

In order to determine the solubility of the delipidated water-insoluble lens fractions, small amounts were suspended in various media. Complete solution was obtained after incubation of samples in 0.1 N NaOH during 5 h at 40°C. The solubility of the delipidated water-insoluble fractions were determined, in relation to alkaline solubility, after incubation of 2 mg samples in 1 ml 7 M urea, 20 mM Tris-HCl, 80 mM NaCl, pH 8.6 (2 and 5 h, 40°C and 1 h, 100°C), in 20 mM Tris-HCl, 80 mM NaCl, pH 8.6 in the presence of trypsin (sample-enzyme weight ratio 50:1) during 2 h at 40°C, in Tris buffer/ 7M urea in the presence of trypsin during 2 h at 40°C and in 0.3 M acetic acid (5 h, 40°C). After incubation under agitation, incubates were centrifuged (3,000 g, 15 min) and protein measured in the supernatants according to the method of Lowry et al. (1951). Corrections were made for absorbances of the blanks. Since DTE interferes with the

Lowry method we could not determine in short time the solubility of delipidated water-insoluble fractions in urea buffer containing DTE.

In order to determine the relative solubility of lens parts in urea buffer, individual decapsulated lenses were separated into cortex and nucleus according to methods described in section 2.2.3. Lens parts were homogenized in 7 M urea, 20 mM Tris-HCl, 80 mM NaCl, 1 mM EDTA (pH 7.3) and the homogenates centrifuged (3,000 g, 15 min) in order to remove urea-insoluble material. The supernatants were adjusted to 2 ml. From this solution 25 µl was taken for the determination of protein according to the method of Lowry et al. (1951). In order to compare these protein data with the wet weight data of the water- and urea-soluble fraction as gathered by Kramps et al. (1976), ratios of water- and urea-soluble protein content of cortices over that of nuclei were calculated.

2.2.9. Enzymic digestions of the yellow urea-DTE-insoluble proteins

The yellow pellet (see section 2.2.7) was treated with proteolytic enzymes in an attempt to concentrate the cross-linked regions of the protein chains, because it is known that such enzymes will not readily split peptide bonds which are adjacent to covalent cross-links.

Digestion with trypsin (carboxymethyl cellulose-bound (CM), Merck 7-10 U/mg) was carried out in 0.1 M ammonium bicarbonate buffer, pH 8.9 during 20 h at 37^{o}C at a protein sus-

pension concentration of 10 mg/ml, using 10% (w/w) of CM-bound enzyme. After the tryptic digestion the mixture was centrifuged (3,000 g, 15 min) to remove the enzyme and any insoluble peptides. The yellow supernatant was lyophilized. The yellow material was digested further with pronase P (from *Streptomyces griseus*, CM-bound, Merck 1.5 U/mg). For this purpose the material was dissolved in 0.05 M phosphate buffer, pH 7.8, at a concentration of 10 mg/ml and a CM-bound enzyme concentration of 1 mg/ml. The digestion was carried out at 37°C during 20 h. After centrifugation a white pellet was obtained containing the enzyme and insoluble material. The supernatant was bright yellow in color.

To the supernatant subtilisin (CM-bound, Merck 7-10 U/mg) was added (1 mg/ml) and digestion continued in the same buffer for 20 h at 37°C. After centrifugation (3,000 g, 15 min) the supernatant was isolated and aminopeptidase M (Röhm, 10 U, 1 mg/ml) was added.

2.2.10. Gel chromatography of the digests

After each digestion step part of the supernatant was lyophilized and applied to a column, containing Bio-Gel P-2 (Bio-Rad Laboratories). This molecular sieve material can be used with water as an eluant. We used a column with an exclusion limit of 1,800 D, 200-400 mesh, and dimensions of 1.3x60 cm. The eluant was distilled water, containing a small amount of preservative (pHix™, Pierce Chem. Co.) and fractions of 3 ml at a flow rate of 18 ml/h were collected at room

temperature. The relative positions of some amino acids were determined. In order to increase the sensitivity of detection, absorbances at 212 nm were measured using a flow-through cell (8 µl) in a Beckmann Model 25 spectrophotometer connected to the column, using water as a reference. Afterwards the absorbances at 212, 280 and 340 nm of every fraction were measured. Relative fluorescence was measured at wavelengths above 435 nm after excitation at 340 nm, using a Zeiss PMQ II spectrophotometer in combination with a fluorescence measuring unit.

2.2.11. Analysis of cross-linked material

After extensive digestion and column chromatography on Bio-Gel P-2 the fractions containing material absorbing both at 280 and 340 nm, and fluorescent above 435 nm (non-tryptophan fluorescence) were lyophilized. Water was removed azeotropically with dichloromethane at 60°C under a stream of nitrogen. The dried yellowish material was derivatized for gas chromatography, following the method as described by MacKenzie and Tenaschuk (1974) with slight modifications.

2.2.12. Gas chromatography

Amino acid standard mixture (2.5 µmol/ml in 0.1 N HCl) was from Beckmann (Palo Alto, California), heptafluorobutyric anhydride (HFBA) was from Pierce (Rockford, Illinois). Isobutanol, dichloromethane and ethyl acetate (Merck, Darmstadt, FRG) were redistilled from an all-glass apparatus after re-

fluxing for 2-6 h over anhydrous calcium chloride or magnesium turnings. Isobutanolic-HCl was prepared by bubbling anhydrous HCl gas through a trap with sulfuric acid and into isobutanol at 0°C until the solution was 3 N in HCl as determined by weighing. Isobutanol-3 N HCl was stored in a nitrogen atmosphere at 20°C. Aliquots were taken by inserting a syringe needle through a rubber septum. To prevent deterioration of HFBA, 200 µl portions were dispensed in ampoules which were sealed after flushing with dry nitrogen and stored at -20°C.

All glassware was heated at 450-500°C for 2-6 h before use. Derivatization was performed in 1 ml Reactivals capped with Teflon Mininert valves (Pierce). The Reactivals were heated during derivatization and evaporation in a Pierce Reactitherm. Samples containing single amino acids or digests were dried at 60°C by inserting a needle through the valve and blowing dry nitrogen through the vial. Subsequently, the last traces of water were removed azeotropically with 100 µl dichloromethane.

For esterification of the amino acids and peptides, 100 µl isobutanol-3 N HCl was added to the vial which was then flushed with nitrogen before closing the valve. The vial was sonicated for 5 min to dissolve the material and then heated at 110°C for 30 min. The sample was dried at 60°C with dry nitrogen and redried after addition of 100 µl dichloromethane.

For acylation, 300 µl ethyl acetate and 100 µl HFBA were added and the vial was heated at 110°C for 20 min. After cooling, the sample was taken just to dryness at room temp-

erature, and redissolved in an appropriate volume of anhydrous ethyl acetate. A Varian Aerograph model 2100 chromatograph equipped with a flame ionization detector and a linear temperature programmer was used. Samples were analysed on a 3.5 m x 3 mm i.d. coiled glass column packed with 3% SE-30 on Gas-Chrom Q, 100-120 mesh (Applied Science Laboratories State College, Pa.). The column was conditioned during 1 h at 320°C and at 270°C with gas flow on overnight.

The chromatographic conditions were: injector temperature, 250°C; detector temperature, 280°C; temperature program: 90°C isothermal for 1 min, then 90-225°C at 4°C/min and finally 255°C isothermal; carrier gas (nitrogen) flow rate, 25 ml/min.

Injection of 1-10 nmol of each derivatized amino acid, dissolved in 1-2 µl ethyl acetate, gave good results.

2.2.13. Combined gas chromatography-mass spectrometry

GC-MS was performed using an LKB-type 9000 GC-MS, using the same chromatographic column as used on the Varian Aerograph. The N-HFB-isobutyl amino acid esters were prepared as described above. The separator oven and transfer line were maintained at 250°C, the ionizer voltage was 70 eV. Spectra were recorded at a rate of 1 sec/decade. The 70-eV electron impact MS fragmentation patterns of the common amino acids (as N-HFB-isobutyl derivatives) have been described recently by MacKenzie and Hogge (1977).

2.2.14. Relationship between lens classification and spectral characteristics of the water-insoluble fraction and separated lens parts

The water-insoluble lens fractions of normal and nuclear-cataractous lenses were used to strengthen the classification scheme. Therefore, equal amounts of the water-insoluble lens fractions were digested according to methods described for yellow protein (section 2.2.9), using only trypsin and pronase P. After centrifugation of the last digestion mixture (3,000 g, 15 min) to remove the enzyme and insoluble peptides, the supernatants were diluted with digestion buffer and absorbances measured at 280 and 340 nm, which are wavelengths typical for aromatic protein residues and non-tryptophan fluorescent compounds, respectively (Lerman and Borkman, 1979).

Embryonic nuclei obtained from nuclear-cataractous human lenses (see section 2.2.3), were digested as described for the water-insoluble protein fraction. Spectral data were normalized on the basis of embryonic nucleus wet weights.

Cortices, embryonic nuclei and remaining nuclear parts from normal and nuclear-cataractous human lenses, varying in age, were homogenized in ice-cold 10% TCA, 2 mM EDTA at a final volume of 2 ml. After centrifugation (3,000 g, 15 min), supernatants were diluted with TCA solution and analyzed on a Perkin-Elmer (Hitachi) 204-A fluorescence spectrophotometer (Xenon 150 watt lamp). After recording of spectra (band widths, 10 nm), fluorescence was measured at 345 nm after excitation at 295 nm (mainly tryptophan fluorescence) and at 415 nm af-

ter excitation at 335 nm (non-tryptophan fluorescence).

2.2.15. Sedimentation analysis

Sedimentation experiments were performed in a Beckman Spinco E analytical ultracentrifuge, equipped with electronic speed control. Sedimentation coefficients were determined near 20°C, using Schlieren optics, from the rate of movement of the maximum of the peak, corrected for the density and viscosity of the solvent relative to water at 20°C ($s_{20,w}$) and extrapolated to zero protein concentration ($s^0_{20,w}$) using linear regression analysis.

2.2.16. Inorganic phosphate determination

Inorganic phosphate was determined according to methods described by Bonting (1970). Samples were dialyzed against water, freeze-dried and further dried over P_2O_5. Lipids were extracted according to Folch et al. (1957). A conversion factor of 25 was used to relate the amounts of phosphate to the amounts of phospholipids.

2.2.17. SDS-electrophoresis

Dodecyl sulfate gel electrophoresis was performed according to Laemmli (1970) with minor modifications (Kramps et al., 1976). Gels were stained with Coomassie Brilliant Blue R250 and destained according to Weber and Osborn (1969). Molecular weights were estimated from a linear plot of log molecular weight versus mobility relative to tracking dye (Bromphenol

Blue) using hemoglobin, ovalbumin, bovine α-crystallin and cytochrome-c as calibration standards.

Separation of lens parts

Schematically (Figure 2.1.) nuclear-cataractous human lenses can be divided into three parts: a transparent, faintly yellow cortex, a highly colored embryonic nucleus, which cracks out itself upon trephinizing the nucleus of this type

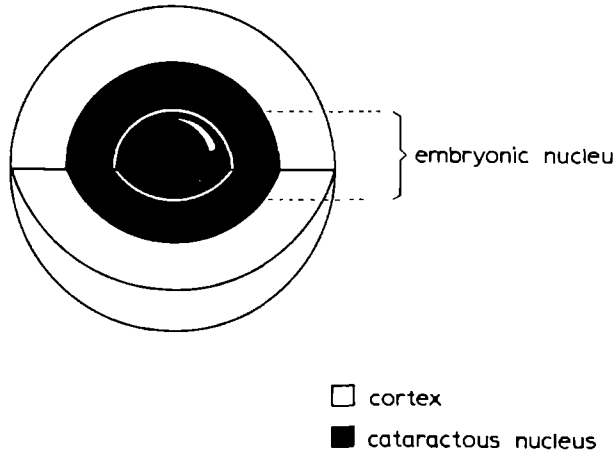


Fig. 2.1. Schematic representation of a human nuclear-cataractous lens

of cataractous lenses, and a colored hull surrounding the embryonic nucleus. The embryonic nucleus is constant in weight (30 ± 2 mg, 10 determinations) and represents about 14% of the wet weight of the adult human lens.

Mechanical-dynamic properties of the lens

One might think that the ocular lens consists of a protein-water gel in which the protein concentration reaches up to 900 mg/ml. As described in Chapter I, slow degradation of polypeptide chains takes place in the aging lens proteins in addition to a process of amino acid deamidation and racemization; these alterations in protein primary structure may result in the formation of new contact regions between lens protein aggregates (Siezen and Hoenders, 1977) or between cytoskeleton, plasma membrane and α-crystallin (Vermorken et al., 1979; Ramaekers et al., 1980). As a result, the distribution of water within the lens fiber cells might change, although the total water content of the aging and nuclear-cataractous human lens remains fairly constant (Kramps, 1977). Conformational changes, partial unfolding and protein insolubilization, eventually accelerated by disulfide and non-disulfide covalent cross-linking, occur to the lens proteins during aging and nuclear cataract formation. The aging lens looses its elastic properties and so does the suspensory system (Farnsworth and Shyne, 1979), as a result the accommodation ability of the human lens is lost at the age of about 50 years. Since most nuclear cataracts also develop at roughly the age of 50 years, there may be a relationship between the loss of mechanical properties of the aging lens and the appearance of nuclear browning. From this point of view it seems worthwhile to shed some light on the mechanical properties of the human lens.

Proteins can form gels in an environment of denaturing agents, if the protein concentration is high enough (Hopkins, 1930). The gel formation of pure proteins seems to be due to the development of intermolecular disulfide bridges (Huggins et al., 1951; Simpson and Kauzmann, 1953; Frensdorff et al., 1953). Recently, it has been shown with rheological measurements that a heatset solution of ovalbumin in 6 M urea behaves like a rubberlike material; from the mechanical-dynamic characteristics of such a gel, information can be obtained about the number of permanent cross-links (van Kleef et al., 1978). Since comparable events, i.e. cross-linking and denaturation of proteins, seem to take place in human lens (for review, cf. Harding and Dilley, 1976), it may be a good source for investigating gel-like phenomena.

The question is raised why the lens is a visco-elastic material, is it because the proteins interact with each other and thus give rise to gel-like behavior or is it because the lens fibers, containing a true protein solution, determine the lens visco-elasticity. To find an answer to these questions, we compared a calf lens protein solution with calf lens cortices.

In Figure 2.2. the frequency dependence of the dynamic moduli for calf lens cortices is shown. From this figure it can be seen that the storage modulus G' is weakly dependent on the frequency, which is in agreement with the fact that the lens is a visco-elastic material. The loss modulus is smaller than the storage modulus. At 2 Hz the storage modulus becomes 120 Pa.

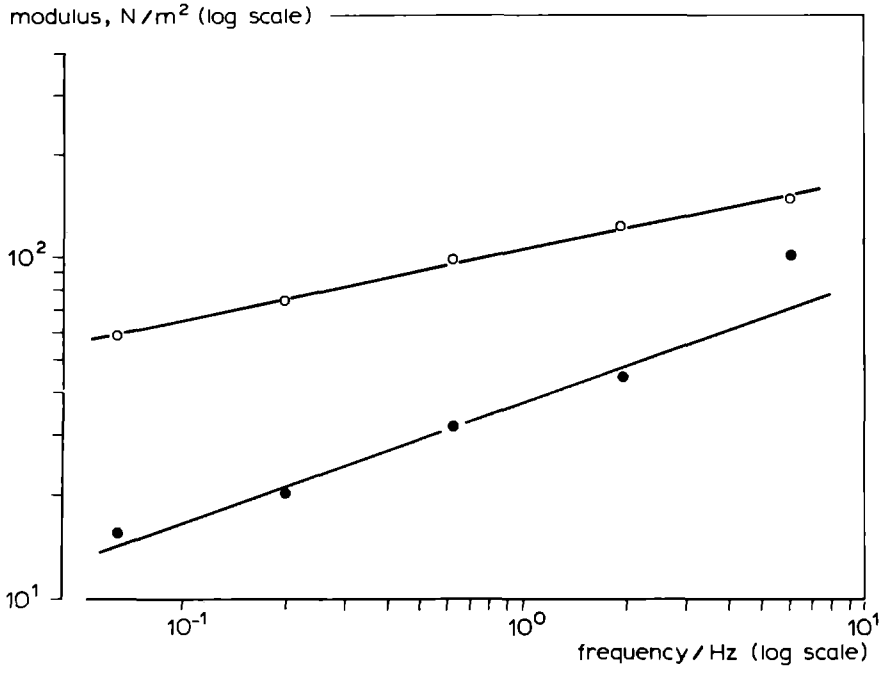


Fig. 2.2. Dynamic moduli as a function of frequency of calf lens cortices, measured at 23°C.
G': storage modulus (o——o), G'': loss modulus (●——●).
Applied deformation: 5×10^{-3} (at the outer rim).

At room temperature, a 30% (w/w) calf lens protein solution shows a storage modulus G' of about 7 Pa, after gelation (by heating during 45 min at 85°C), it becomes 10^5 Pa (Table 2.I).

From the comparison between the protein solution and the calf lens cortices, we can conclude that the protein solution in the lens does not make a contribution to the mechanical properties of the lens.

Due to lack of human lenses and due to complete loss of material using rheological measurements only few lenses could be spent for investigation. At 2 Hz, a normal human lens (65

TABLE 2.I.

MECHANICAL-DYNAMIC PROPERTIES OF LENSES AND LENS PROTEIN SOLUTIONS

	Normal lens (65 yrs)	Nuclear-cataractous lens (67 yrs)	Cortex calf lens	30% (w/w) calf lens protein	30% (w/w) solution of calf lens protein after gelation
G' (2 Hz) N/m^2	20,000	38,000	120	7	100,000
G''/G' (tg δ)	0.4	0.3	0.4	1	0.1

years) shows a storage modulus of 2×10^4 Pa and a nuclear-cataractous lens (brown nucleus, 67 years) of 3.8×10^4 Pa (Table 2.I.).

The frequency dependence of the dynamic moduli of a decapsulated normal and nuclear-cataractous lens was determined (not shown). The storage modulus of the cataractous lens turned out to be greater than that of the normal lens; the frequency dependence of the moduli was the same and similar to that of calf lens cortices (see Fig. 2.3.). The ratio G'/G'' was about the same for both lenses (0.4 and 0.3, resp.).

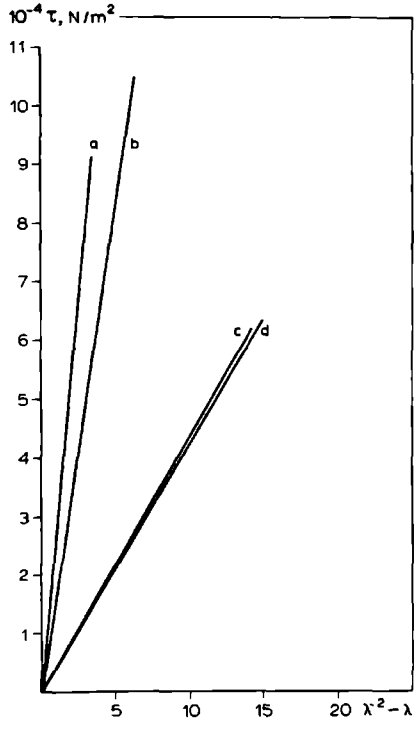


Fig. 2.3. Relationship between stress and compression of normal and nuclear-cataractous lenses. (a) Nigra, 76 yrs; (b) brown, 65 yrs; (c) normal, 51 yrs; (d) normal, 51 yrs, decapsulated.

As a conclusion, the lenses show the same mechanical properties, i.e. they behave both like visco-elastic materials. From the compression measurements (Fig. 2.3.) it can be deduced that nuclear-cataractous are about four times as hard as normal lenses of the same age. This fact makes it feasible to isolate the hard nucleus from nuclear-cataractous lenses, whereas in normal lenses this nucleus keeps rather soft during lifetime.

To summarize, we can conclude that the dissolved protein solution in the lens (in the experiment a concentration of 300 mg protein/ml was used) does not contribute much to the mechanical properties of the lens. The visco-elastic properties of the lens seem to be primarily determined by the lens fiber membranes. Upon aging and, in particular, upon nuclear cataract development, plasma membranes of the inner region of the lens show degeneration (Kobayashi and Suzuki, 1975), which process changes the mechanical-dynamic properties of the lens nucleus, leading to presbyopia, concomitantly with a decrease in zonular fiber elasticity at old age. More information about the elastic behavior of the human lens has been given by Fisher 1971, 1973a,b). It can be further concluded that nuclear-cataractous lenses are much harder than normal lenses, hardness is caused by the nuclear region of the lens making it feasible to isolate this part of the lens according to methods described in section 2.2.3.

Designing an isolation scheme for lens protein fractions

When homogenates of pooled normal lenses, ranging in age from 50 to 80 years, are centrifuged consecutively at variable centrifugation forces and the supernatants are applied to a Bio-Gel A-5m column, elution patterns are obtained, parts of which are depicted in Figure 2.4. Examination of the

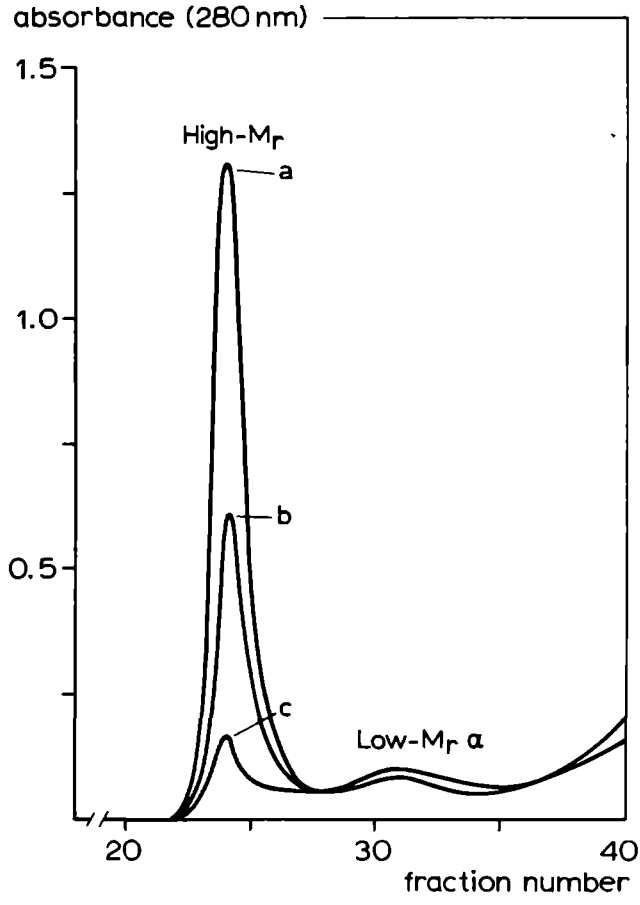


Fig. 2.4. Size fractionation on an agarose Bio-Gel A-5m column (2.5x100 cm; flow rate, 0.32 ml/min). The elution pattern after low-M_r α-crystallin has been omitted.
a) Total 9,900 g, 25 min supernatant; b) total 26,000 g, 55 min supernatant; c) total 100,000 g, 55 min supernatant

protein content of the first two peak fractions, representing high-M_r proteins and low-M_r α-crystallin, respectively, reveals that high-M_r proteins are nearly absent from the supernatant of lens homogenates centrifuged at the highest force (100,000 g, 55 min). Although human high-M_r proteins are not completely composed of α-crystallin, in majority they are and, therefore, we can calculate the expected protein content of the high-M_r fraction, using $E^{1\%}_{280} = 15.6$ for α-crystallin as given by Lerman and Borkman (1978a). If absence of light-scattering is assumed, less than 75% of the absorbing protein eluting in the high-M_r fraction represents true protein on the basis of the Lowry protein determination method. However, since these molecules have apparent molecular weights over 5×10^6 daltons, scattering may attribute over 50% to the absorbance at 280 nm. Therefore, the high-M_r fraction cannot represent pure protein.

High-M_r proteins, isolated as pellet at 100,000 g, 55 min from pooled normal and nuclear-cataractous lenses (10 lenses of each type), contain on a dry weight basis up to 60% (range 42-62%) protein in nuclear-cataractous lenses and 75% in normal lenses. It can be concluded from these findings that in nuclear-cataractous lenses less than 60% of the high-M_r protein fraction, isolated as pellet at 100,000 g, 55 min represents protein, whereas in normal lenses more protein may be spun down at this force. The remaining material can not represent protein and even no 280 nm-absorbing material, since much of the absorbance at 280 nm by the high-M_r proteins

can be accounted for by light-scattering. Lipids might be attached to the high-M_r proteins in amounts becoming somewhat greater upon development of nuclear cataract. These findings may represent artefactually formed protein-lipid vesicles, since, as we shall discuss in Chapter VIII, nuclear cataractous lenses may contain relatively much lysophosphatidyl ethanolamine (LPE) in the water-soluble (9,900 g supernatant) lens fraction. On the other hand, strong interaction of newly-synthesized lens proteins with plasma membranes has been found (Ramaekers et al., 1980), interaction between which is mainly due to α-crystallin A_2-chains. It is safe to say that after consecutive centrifugation at 9,900 g during 25 min and at 100,000 g during 55 min more than 90% of all high-M_r proteins are removed from the water-soluble lens fraction, containing, amongst others, lipid components.

Urea-DTE-soluble proteins

When individual human nuclear-cataractous lenses are homogenized in urea/buffer, containing DTE, and insoluble material (mainly yellow proteins and plasma membranes) is removed by centrifugation, elution patterns of the solubilized proteins on Bio-Gel A-5m (in urea-DTE/buffer) are obtained as depicted in Figure 2.5. Under these conditions lens proteins are thought to be fully dissociated and disulfide bonds reduced. It has been postulated (Harding and Dilley, 1976; Kramps et al., 1978c) that the first fraction, eluting from the column may represent material that is soluble in urea/buffer, but is held together by non-disulfide covalent bonds. Increasing

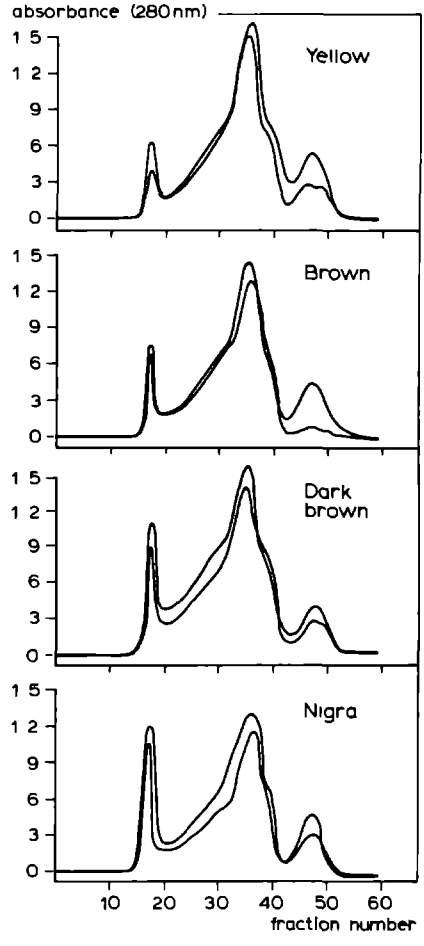


Fig. 2.5. Size fractionation on an agarose Bio-Gel A-5m column (1.6x70 cm; flow rate, 0.15 ml/min) of urea-DTE-soluble proteins from individual nuclear-cataractous human lenses. Color of the lens nucleus is indicated.

amounts of these high-M_r proteins (first peak) are found in relation to lens nuclear color deepening, suggesting a cataractous state-dependent process. Omitting DTE during the elut-

ion procedure (not shown) does not change the patterns. The material eluting in the first fraction cannot be further analyzed using electrophoretic techniques since it does not penetrate polyacrylamide gels commonly used for molecular weight and isoelectric point estimations (Kramps et al., 1978). The molecular weights of gel-penetrating proteins (fraction numbers 25-40) have been tabulated (Table 2.II) for nuclear-cataractous lenses. Most protein bands are rather diffuse.

TABLE 2.II

APPARENT MOLECULAR WEIGHTS OF POLYPEPTIDE CHAINS FROM UREA-DITHIOERYTHRITOL-SOLUBLE FRACTIONS FROM NUCLEAR-CATARACTOUS HUMAN LENSES.
Three determinations on each sample were performed; mean data are tabulated. Diffuse bands are given in parentheses.

Apparent molecular weight	Assigned to*+
(6,500-12,500)	
(11,000)	gamma
17,500	gamma, beta
19,000	alpha?
20,000	alpha
22,000	alpha
24,000	beta
26,500	membrane protein (MIP)∞
27,500	
29,000	beta
30,500	
(37,500)	
46,000	extrinsic membrane protein∞
(52,000)	
(60,000-65,000)	
(>100,000)	not penetrating

*If not stated otherwise, crystallins are meant.
+Given by Kramps et al. (1976), ∞Given by Spector et al. (1979).

Sedimentation analysis of the firstly eluting fractions from the Bio-Gel A-5m column revealed that they contain proteins with sedimentation values of 270S and higher (compare with α-

crystallin: 19.5 ± 0.9 S, 16 determinations), besides some flotating material. The first fractions contained 16 µg phospholipid/mg dry weight and negligible amounts of protein-bound phosphate, whereas the total material applied to the column contained 7.4 µg phospholipid/mg material. It is evident from these findings that protein-lipid complexes have been formed, which contain cross-linked proteins. These findings may point to an increased imbibement of cross-linked proteins in the plasma membranes and/or to an increase in hydrophobicity of unfolded and cross-linked proteins, which process may result in increased association of the proteins with lipids. Segregation of membrane lipid components may take place upon aging and nuclear cataract development *in vivo*, as will be discussed in Chapter VIII.

It cannot be deduced from our findings at which solubility state of lens proteins the processes mentioned have taken place. It has been suggested that human lens proteins become water-insoluble, but urea-soluble during aging of the lens and eventually become even urea-insoluble during nuclear cataract development (Roy and Spector, 1978c). Kramps et al. (1978c) have shown that even omission of DTE from urea/buffer solution, used to homogenize the lens, did not change their findings with respect to the high molecular weight urea-soluble proteins, which are non-disulfide covalently cross-linked; they concluded that the proteins, which contained no non-disulfide covalent cross-links might contain intramolecular disulfide bonds, although the presence of bonds between these proteins and low

molecular weight compounds, like glutathione, could not be denied.

Solubility of nuclear-cataractous human lens fractions

The relative solubility of water-insoluble fractions from nuclear-cataractous human lenses are given in Table 2.III.

TABLE 2.III

SOLUBILITY OF DELIPIDATED WATER-INSOLUBLE FRACTION FROM NUCLEAR-CATARACTOUS LENSES
The number of determinations are given in parentheses; values are expressed as mean ± SD.

Solubilization procedure*	Protein solubilized* (%)
0.1 N NaOH, 5 h, 40°C	100
7 M Urea, 20 mM Tris-HCl, pH 8.6	
40°C, 2 h	38.7 ± 5.3 (2)
40°C, 5 h	64.1 ± 2.1 (2)
100°C, 1 h	43.7 (1)
20 mM Tris-HCl, pH 8.6, trypsin, 2h, 40°C	18.0 ± 1.3 (2)
IDEM + 7 M urea	44.0 ± 0.5 (2)
0.3 M Acetic acid, 5 h, 40°C	12.0 ± 1.0 (2)

*Samples (2 mg/ml) were incubated during indicated periods, centrifuged and protein determined in the supernatants.
*Protein was measured by the Lowry method.

Apparently, 39% of the proteins in the water-insoluble fractions can be solubilized in urea/buffer solution after 2 h incubation at 40°C. Raising the temperature (100°C) does not lead to increased solubilization, whereas longer incubation at 40°C may result in a higher percentage solubilization. It should be kept in mind that this procedure is rather arbitrary

since longer incubations of lens protein samples in urea/buffer (pH 8.6) may induce disulfide bonds or blocked sulfhydryl groups, although more protein may be solubilized by longer treatment.

In order to isolate the yellow protein without risking sulfhydryl modification, delipidated water-insoluble fractions from nuclear-cataractous lenses might be better extracted with urea/buffer (pH 7.3) in the presence of DTE. Since DTE interferes with the Lowry method, we could not determine in short time the relative solubility of delipidated water-insoluble fraction in urea/buffer (pH 7.3) containing DTE. On a dry weight basis, 6 to 7% of the total delipidated lens material remains insoluble in urea/buffer containing DTE at the most severe states of cataract, this fraction represents the yellow protein fraction.

Comparing the protein contents of the water- and urea-soluble fraction from cortices and nuclei of individual lenses (expressed as ratio) with the wet weight of this fraction (Kramps et al., 1976, pooled lenses), it can be shown that decreasing amounts of protein can be solubilized in urea/buffer (without DTE) from the nuclear part of these lenses in relation to the cataractous state (Table 2.IV). During nuclear cataract development increasing amounts of non-protein material accumulate in the water- and urea-soluble fraction of the lens nucleus, this material might be composed of lipids derived from nuclear plasma membranes as was indicated by the findings for the urea-DTE-soluble protein fraction.

TABLE 2.IV

COMPARISON OF RATIOS OF PROTEIN CONTENT WITH TOTAL WET WEIGHT DATA OF THE WATER- AND UREA-SOLUBLE FRACTION FROM CORTICES AND NUCLEI OF NORMAL AND NUCLEAR-CATARACTOUS HUMAN LENSES OF COMPARABLE AGE.
Values are expressed as mean ± S.D. The number of determinations are given in parentheses.

Nuclear color	Ratio protein data Co/Nu	Ratio wet weight data Co/Nu*
Normal	1.35 ± 0.46 (14)	0.8 (1)
Yellow	1.53 ± 0.79 (11)	1.3 (1)
Brown	1.67 ± 0.56 (8)	1.5 (1)
Dark brown	2.40 ± 0.65 (7)	1.8 (1)
Nigra	2.56 ± 1.76 (7)	2.2 (1)

*Taken from Kramps et al. (1976) and Kramps (1977)

Whereas the ratios of wet weight data from cortices and nuclei increase about threefold during nuclear cataractogenesis, the ratios of protein contents only increase twofold. This finding might imply that during nuclear cataract maturation more and more membrane material is isolated in the water- and urea-soluble fraction, being associated with lens proteins.

Yellow protein fraction

The characteristic features of senile nuclear-cataractous lenses appear to be the coloration and insolubilization of lens proteins, centered primarily in the nuclear region of the lens (Dilley and Pirie, 1974). More precisely, the alterations which take place in the nuclear-cataractous lens have been assessed by a) formation of urea-insoluble and urea-DTE-insoluble proteins and protein-lipid complexes, b) increase in the UV absorption at 325-355 nm, c) development of non-tryptophan fluorescence at wavelengths over 400 nm. The inso-

lubility of the urea-insoluble fraction of the cataractous nucleus is believed to be caused by disulfide bonds between and/or within the polypeptide chains (Harding and Dilley, 1976; Kramps et al., 1976). This fraction, however, contains a considerable amount of lens fiber membranes, in addition to granular material (Maisel, 1977). After extraction of the membrane components with organic solvents according to Folch et al. (1957), there remains, however, proteinaceous material which is resistant to the dissociating medium, containing urea and reducing agent (Buckingham, 1972). The amount of this material increases with increasing nuclear color (Kramps, 1977). It seems to have a very high molecular weight, which may be the result of both aggregation of smaller subunits and formation of non-disulfide covalent bonds, as deduced from the experiments described earlier (Pp. 93-97). As the major site of the pigmentation and fluorescence of the senile lens is the insoluble, non-reducible fraction (Augusteyn, 1975; Zigman et al., 1976a), these cross-links may be responsible for the aggregation (up to insolubilization) and coloration of the nuclear proteins; the cross-links may be considered to be part of the final stage in the development of brown nuclear cataract. In the following experiments we tried to isolate and identify the cross-links being part of the yellow protein fraction. Preliminary experiments on cross-link enriched fractions will be described in this chapter, whereas the relationship between the development of cross-links and the stage of nuclear cataract will be described in Chapter III.

In order to avoid the presence of lipids in our yellow protein samples, we delipidated these fractions before further characterization. Applying, however, a modified Folch-routine (Folch et al., 1957) to the water-insoluble fractions, proteins were isolated as one band. This procedure was further omitted, since much of the protein in this band could not be yellow as judged by the color appearance of the band. Since we were interested in isolating rather pure protein devoid of membrane components, a delipidation method was applied to the water-insoluble lens fractions, as described by Cham and Knowles (1976). According to this method, proteins may be mildly delipidated in the presence of buffer solution. After the extraction procedure negligible amounts of phosphate could be detected in the yellow protein fraction.

After trypsin-pronase P digestion of the yellow protein fraction from pooled dark brown nuclear-cataractous lenses, the soluble part of the digest shows the UV-visible spectrum as shown in Figure 2.6., which is in good agreement with the

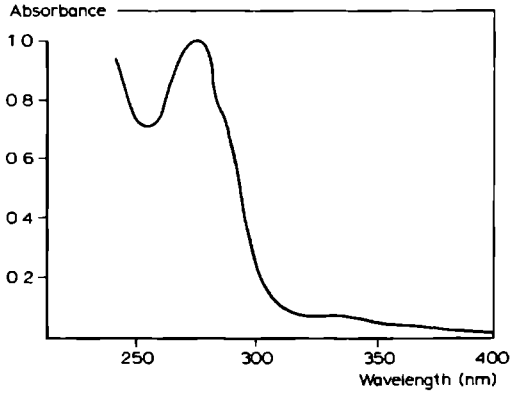


Fig. 2.6. Absorbance spectrum of the soluble part of the trypsin-pronase P digest of the urea-DTE-insoluble protein from the dark brown cataractous lens nucleus. The dilution is arbitrarily chosen.

spectrum given by Dilley and Pirie (1974). Since the material is digested into small fragments, no strong light-scattering is assumed. It can be seen that there is a weak absorption at 340 nm, which may be attributed to a fluorophor, emitting at wavelengths above 435 nm (non-tryptophan fluorescence).

The elution patterns on Bio-Gel P-2 of both untreated and sodium borohydride-reduced tryptic digests of the yellow material are shown in Figures 2.7 and 2.8. Qualitative examina-

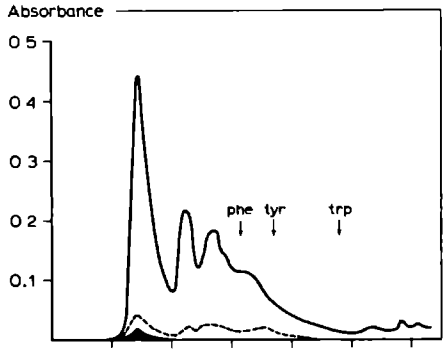


Fig. 2.7. Elution profile of the soluble part of the tryptic digest of the urea-DTE-insoluble protein from the dark brown cataractous lens nucleus. Fractionation was performed on Bio-Gel P-2, equilibrated with distilled water (column dimensions, 1.3x60 cm, flow rate 18 ml/h). Fractions of 3 ml were collected and their absorbances measured at three wavelengths. Absorbance (A) at 212 nm (——), at 280 nm (---), at 340 nm (■). Experimental conditions are described in detail in the text. Relative positions of Phe, Tyr and Trp are indicated.

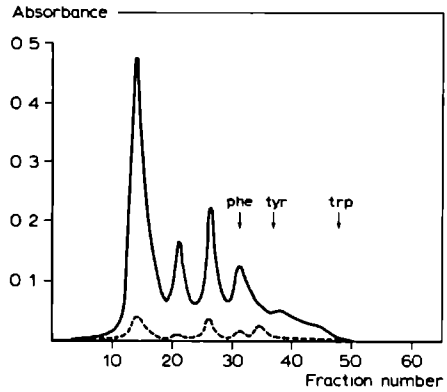


Fig. 2.8. Elution profile of the soluble part of the tryptic digest of the urea-DTE-insoluble protein from the dark brown lens nucleus after previous reduction with sodium borohydride. Fractionation was performed on Bio-Gel P-2. For details see Figure 2.7.

tion of the elution peaks in both figures indicates that only in the void volume (fraction numbers 13-15) material is present, absorbing both at 280 and 340 nm; this material can be reduced by borohydride as indicated by the disappearance of the 340 nm absorption. This may imply an iminopropene structure, being part of the cross-link.

After trypsin-pronase P digestion, a white material, comprising 5-10% of the starting material, remained insoluble; this may represent a so-called core of insoluble hydrophobic peptides that are not digestable by the enzymes used or simply peptides that are insoluble at the pH used for digestion. The colorless material may also attribute to the insolubility of the yellow protein fraction in addition to the effect of the cross-links. It seems reasonable to assume that the cross-links are located in the solubilized (digested) part of the protein fraction, causing its yellow color.

The elution pattern obtained after trypsin-pronase P-aminopeptidase M digestion of the yellow material (Figure 2.9a) shows that there are two fractions which reveal non-tryptophan fluorescence after excitation at 340 nm (Figure 2.9b). The void volume fractions contain incompletely digested material; after lyophilization of these fractions (numbers 13-15) and digestion with subtilisin, material eluting at fraction numbers 23-27 (Figure 2.10) can be separated, which seems to be the same material as already could be isolated in fraction numbers 23-27 (Figure 2.9a). It seems reasonable to assume that these fractions are the same as far as the cross-linked material is concerned. Fract-

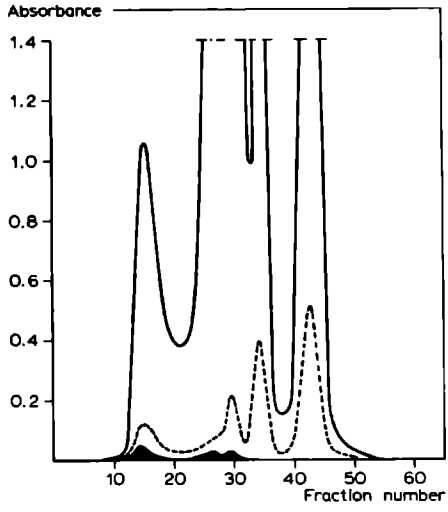


Fig. 2.9a

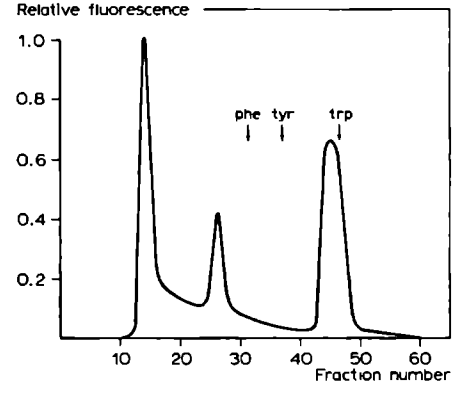


Fig. 2.9b

Fig. 2.9a Elution profile of the trypsin-pronase P-aminopeptidase M digest of the urea-DTE-insoluble protein from the dark brown cataractous lens nucleus. Fractionation was performed on Bio-gel P-2. For details see Figure 2.7. Fractions 13-15 were pooled for further digestion.

Fig. 2.9b Relative fluorescence of the column effluent shown in Figure 2.9a (excitation at 340 nm, emission above 435 nm).

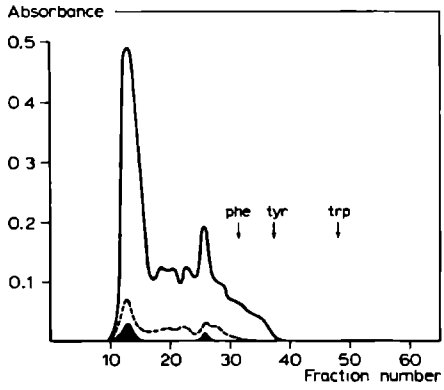


Fig. 2.10. Elution profile of the subtilisin digest of fractions 13-15, isolated as shown in Figure 2.9a. For details see Figure 2.7. Fractions 23-27 were pooled and analyzed by means of GC-MS.

ions 23-27 were analyzed by means of GC-MS, following methods described. In Figure 2.11a, the gas chromatographic pattern of a standard amino acid mixture is shown. Components appearing at 220°C and higher are also found in the reagents blank (see also Table 2.V).

Table 2.V. RETENTION TEMPERATURE OF N-HFB-ISOBUTYL ESTERS OF AMINO ACIDS

Amino acid	Abbreviation	Temperature °C	
Alanine	Ala	112.5	
Glycine	Gly	125	
Valine	Val	135	
Threonine	Thr	137	
Serine	Ser	138.5	
Leucine	Leu	141.5	
Isoleucine	Ile	143	
Proline	Pro	151.5	
Methionine	Met	165.5	
Asparagine/aspartic acid	Asp	172	
Phenylalanine	Phe	176.5	
Glutamine/glutamic acid	Glu	184.5	
Lysine	Lys	191	
Tyrosine	Tyr	193	
Arginine	Arg	202.5	not shown
Histidine	His	207	not shown
Tryptophan	Trp		not shown
Cystine	Cys	239	not shown

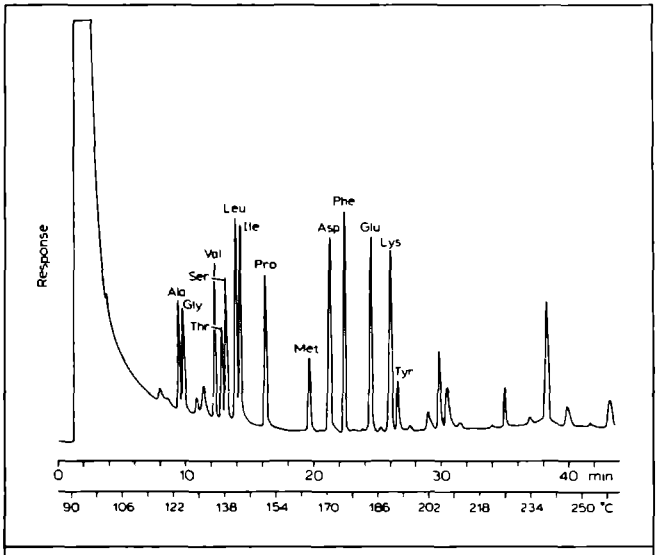


Fig. 2.11a

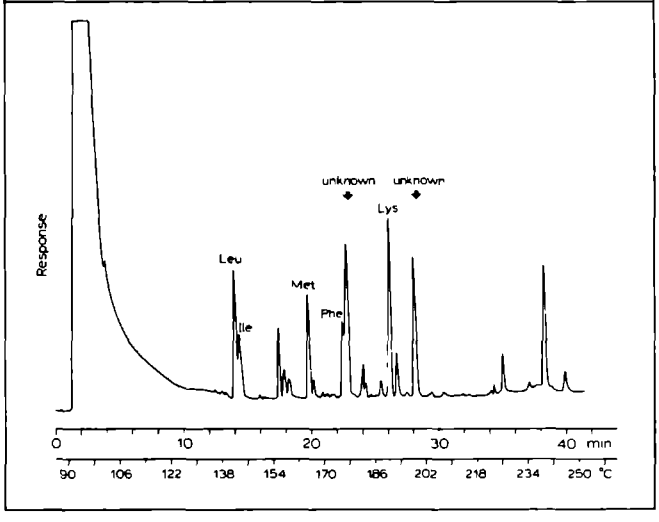


Fig. 2.11b

Fig. 2.11. Gas chromatographic separation of the N-HFB-isobutyl esters of amino acids on a 3.5 mx3 mm glass column packed with 3% SE-30 on Gas-Chrom Q. Temperature program: 1 min isothermal at 90°C, followed by 4°C/min to 255°C, and then isothermal at 255°C. The sample was injected in 2 µl ethyl acetate. *a.* Common protein amino acids. *b.* Pooled fractions 23-27 from the Bio-Gel P-2 column (the trypsin-pronase P-subtilisin-aminopeptidase M digest of the urea-DTE-insoluble protein from the dark brown cataractous nucleus, after derivatization). For abbreviations and details see Table 2.V.

As can be seen in Figure 2.11b, the derivatized pooled fractions 23-27 from the Bio-Gel P-2 column contain some known amino acids (Lys, Met, Ile, Phe) and two or more unknown compounds. Using authentic aminoethylated cysteine or a hydrolyzate of aminoethylated α-crystallin, the peak eluting near 220°C turned out to be aminoethylated cysteine (see also Siezen and Magus, 1977). Another unknown fraction, eluting near Phe (see Fig. 2.11b) was not always present but has been tentatively assigned to ornithine, which could be demonstrated to be present on the amino acid analyzer, after overloading the column with acid hydrolyzed yellow protein. Peaks eluting before methionine could be assigned to oxides of this amino acid by reference to standard compounds. Whether these substances are formed during isolation or during the derivatization procedure remains to be elucidated (see Chapter VII). Sometimes, a peak was observed eluting before alanine, which could be assigned to cysteic acid. This peak disappeared after standing of the derivatization mixture. Whether the cross-link is hydrolyzed during the derivatization step preceding the gas chromatographic analysis could not be deduced from our findings; after sodium borohydride reduction of yellow protein and gel chromatography, no obvious changes could be found in the gas chromatographic patterns. It is concluded from these experiments that the fraction numbers 23-27 contain "brown" peptide fragments which are resistant to further digestion by proteolytic enzymes; these fragments may be not volatile enough to be seen on the analytical part

of the gas chromatographic pattern. Material eluting from the Bio-Gel column at numbers 23-27 and on the GC column did not reveal any analyzable mass spectra, except for normal amino acids.

Changing the derivatization method (N-TFA methylesters, according to Islam and Darbre, 1972) did not give better results, although we could make use of computerized data. Analysis of pooled fractions 23-27 by means of the DNS-Cl method (Woods and Wang, 1967) gave some clues concerning the assumption that they contain some incompletely digested peptides, blocked or non-derivatizable amino groups. It may be mentioned that Kramps (1977) investigated the presence of collagen-like cross-links, isopeptide bonds and bityrosine bonds in several lens protein fractions but, so far all investigations gave negative results.

Lens nuclear color and spectral characteristics of lens fractions

After digestion of water-insoluble proteins (9,900 g pellets) from normal and nuclear-cataractous lenses (10 lenses from each type), a relationship is obtained between the spectral characteristics (ratio 280/340 absorbances) of the solution obtained and lens nuclear color classification, as performed by the ophthalmologist (Fig. 2.12). If we assume that no decreases occur in the tryptophan and tyrosine content of the water-insoluble fractions upon nuclear cataractogenesis,

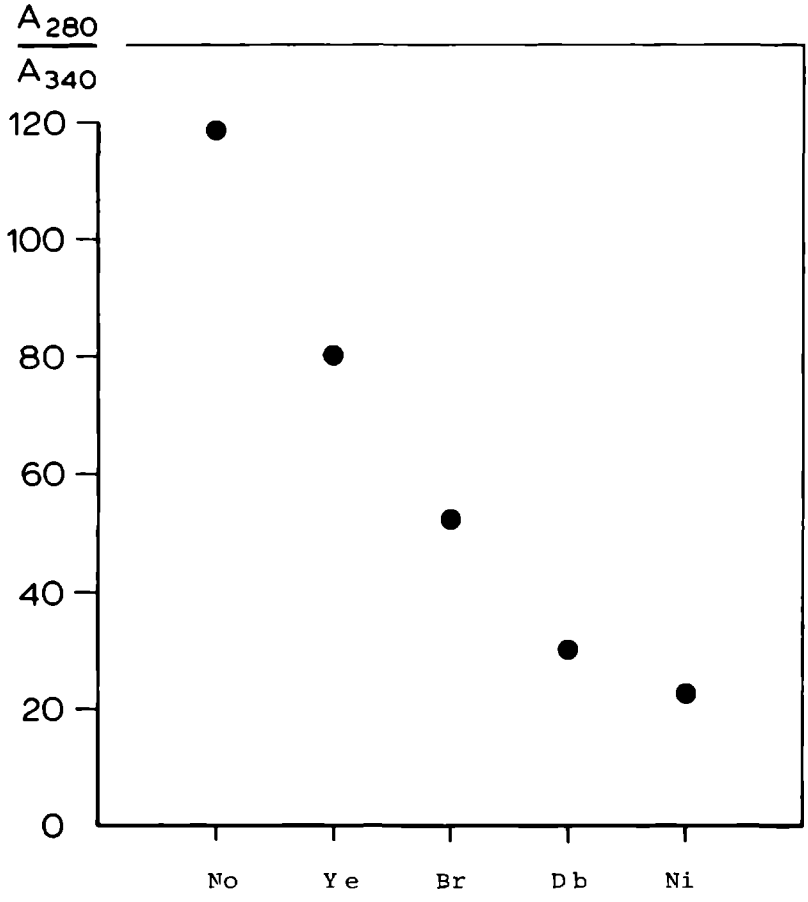


Fig. 2.12. Relationship between the ratio of absorbances at 280 over 340 nm of digests of water-insoluble fractions derived from normal and nuclear-cataractous lenses and lens nuclear color classification.

this figure represents the relationship between accumulation of non-tryptophan fluorescent, 340 nm-absorbing components in the water-insoluble protein fraction and nuclear color. We shall return to these subjects in Chapters III and IV.

After digestion of embryonic nuclei from nuclear-cataractous lenses, a normalized scatter diagram is obtained (Fig.

2.13), revealing a relationship between spectral data of lens parts and color classification of the lens; this diagram is

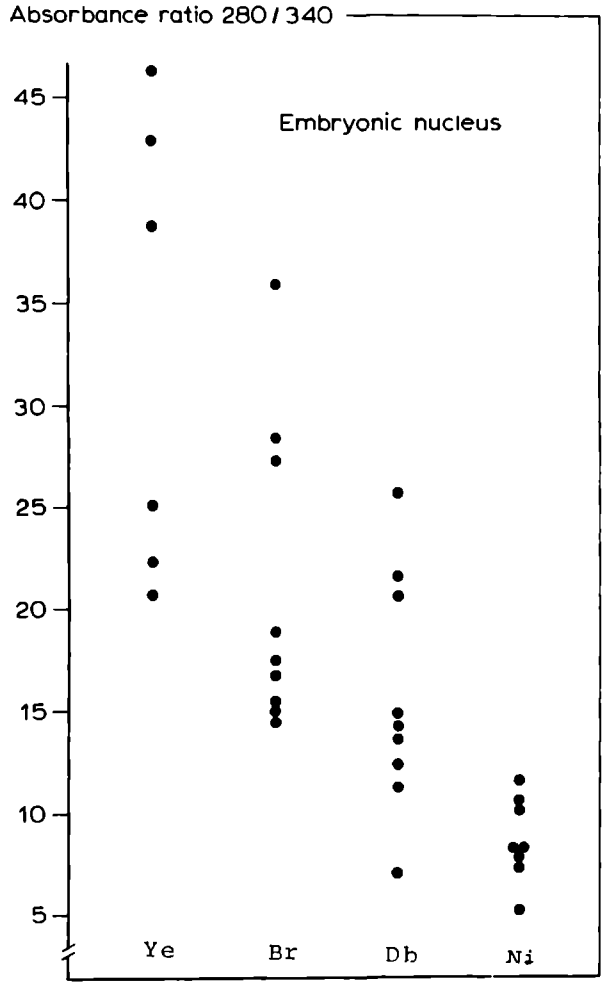


Fig. 2.13. Relationship between the ratio of absorbances at 280 over 340 nm of digests of embryonic nuclei derived from nuclear-cataractous lenses and lens nuclear color classification.

very similar to that depicted in Figure 2.13. From these two figures (2.12 and 2.13) and from the findings on lens protein distribution, obtained by Kramps et al. (1976), it may be concluded that our classification scheme, dividing nuclear-cataractous lenses into groups of increasing nuclear color is reflected satisfactorily by biochemical and spectral data.

When comparing relatively young material (cortex) with the oldest part of the lens (the embryonic nucleus), it becomes evident that non-protein-bound fluorescent compounds can be divided into two groups (Fig. 2.14): non-tryptophan fluores-

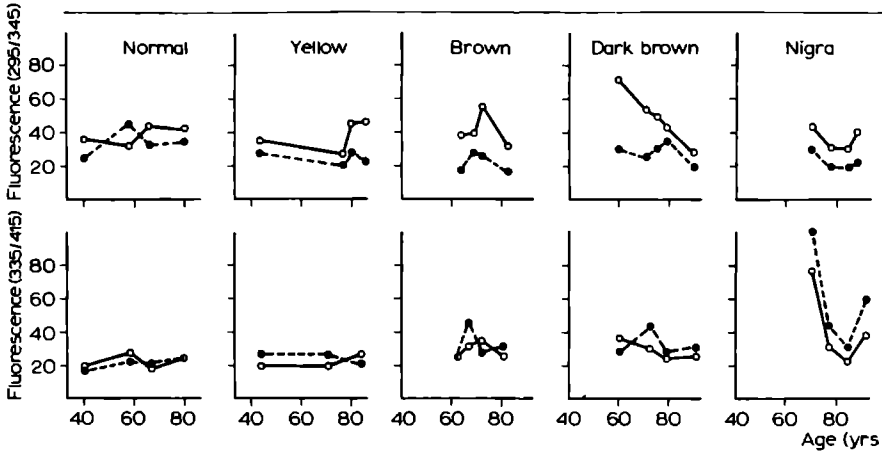


Fig. 2.14. Relationship between (normalized) fluorescence data derived from trichloroacetic acid soluble fractions from cortices and embryonic nuclei of normal and nuclear-cataractous lenses and lens age. 295/345 represents tryptophan fluorescence (mainly). 335/415 represents non-tryptophan fluorescence.

o—— o Cortex data; ●---● embryonic nucleus data.

cent compounds, which seem to accumulate in the inner region of the lens (dotted line above solid line) and tryptophan-fluorescent compounds, which are abundant in the cortical region, but which seem to decrease in concentration going from cortex to embryonic nucleus. More about these findings will be discussed in Chapter IV.

CHAPTER III

DEVELOPMENT OF NON-DISULFIDE COVALENT CROSS-LINKS IN HUMAN LENS NUCLEAR CATARACTOGENESIS 116

3. DEVELOPMENT OF NON-DISULFIDE COVALENT CROSS-LINKS IN HUMAN LENS DURING NUCLEAR CATARACTOGENESIS

3.1. Introduction

Human nuclear-cataractous lenses are divided into four groups of increasing nuclear color. These groups can be considered as successive stages in the development of nuclear cataract; this hypothesis was further strengthened by experiments described in Chapter II. An isolation pathway for human lens fractions and individual proteins, based on introductory experiments (Chapter II) and on literature data has been designed.

In nuclear-cataractous lenses a protein fraction is present, which does not dissolve in 7 M urea, containing reducing agent (DTE). In the urea-DTE-soluble fraction of these lenses proteins are present, which are not dissociated into subunits. Two pathways may be put forward along which the phenomena occurring to the protein in these lenses can be described:

a) in the water-soluble lens proteins non-disulfide as well as disulfide covalent cross-links may develop, making them water-insoluble, but urea-soluble and eventually urea-insoluble; b) in the water-insoluble lens fractions, which may age-dependently increase in weight percentage, non-disulfide covalent cross-links may develop as an ultimate stage in a process of protein denaturation, depending on the number of disulfide and non-disulfide cross-links, the proteins in this fraction may be urea-soluble or urea-insoluble. Whether the

color of the urea-DTE-insoluble protein fraction is associated with the non-disulfide covalent cross-links, remains to be elucidated.

In this chapter, the results from investigations on the yellow, urea-DTE-insoluble protein fraction from nuclear-cataractous human lenses after digestion with several proteolytic enzymes are described. Non-disulfide covalent cross-links, which may be colored and/or fluorescent are purified by means of high pressure liquid chromatography. Further analysis is performed using gas chromatography, amino acid analysis and mass spectrometry. The evolvement of identified compounds from natural precursors will be discussed in relation to human lens nuclear cataractogenesis.

3.2. MATERIALS AND METHODS

3.2.1. Lenses

Human lenses were obtained and handled as described in Chapter II, section 2.2.1.

3.2.2. Isolation of yellow proteins (standard method)

Based on introductory experiments described in the preceding chapter and on literature data, an isolation pathway was designed by which lens fractions including the yellow proteins could be isolated in shortest time. For reasons to be explained in the Results and Discussion no protein modification was performed, only dithioerythritol (DTE) and EDTA were used to prevent sulfhydryl oxidation and proteolysis, respectively. In order to prevent conformational changes in proteins and disulfide bond formation, lyophilization was omitted until the final isolation steps.

Yellow protein was isolated from the non-lyophilized water-insoluble fractions of nuclear-cataractous lenses (10 pooled lenses from each color state) according to a scheme depicted in Scheme 3.1. The water-insoluble fractions were delipidated as described in section 2.2.7 without lyophilization step.

3.2.3. Enzymic digestion of yellow, urea-DTE-insoluble proteins

Digestions were performed according to methods described in section 2.2.9, with the exception that 0.1 M NH_4HCO_3 (pH 8.0) was used as digestion 'buffer' throughout. Digests were covered

with two drops of toluene to prevent bacterial growth. Solvents were purged through Selectron filters (type AE95, pore size 1.2 µm, Schleicher and Schüll, FRG) using a Millipore holder (Millipore, USA).

3.2.4. Gel chromatography of the digests

After the digestion, the final supernatants were purged through a Selectron filter (see section 3.2.3) and 2 ml of each supernatant were applied to a Bio-Gel P-2 column. Digestion buffer was used as an eluant. Transmittance at 280 nm was measured using a Uvicord II photometer.

3.2.5. Analysis of the digests by means of high pressure liquid chromatography

After extensive digestion and column chromatography on Bio-Gel P-2, the fractions containing material absorbing both at 280 and 340 nm and fluorescent above 435 nm (non-tryptophan fluorescence), were analyzed by means of high pressure liquid chromatography (HPLC). A Lichrosorb-10RP18 (Merck, FRG) reversed-phase column (25x4.6 cm i.d.) was used. Water-methanol mixtures were used as eluants at a flow rate of 1.5 ml/min, maintained constant at an average backpressure of 20 MPa by means of an Orlita pump (DMP-AE 10.4.4). Detection was performed using a Pye Unicam LC3 variable wavelength spectrophotometer (Philips, The Netherlands), equipped with an 8 µl flow-through quartz cell (optical pathlength, 1 cm). Samples were injected, using a Glenco syringe (Houston, Texas), via a Valco injection

valve (CV 6-UHPA) equipped with a 10 µl loop. In some experiments, whole digests were applied to a Partisil-10 straight-phase column (25x4.6 cm i.d., Chrompack, The Netherlands) and eluted with a mixture of n-butanol: acetic acid: water (5:2:3, v/v/v).

3.2.6. Preparative high pressure liquid chromatography

For preparative purposes, whole digests were lyophilized and the dry material taken up in small volumes of eluant used for HPLC. Solubilization was aided by sonication. A Lichrosorb-10RP18 column (25x0.9 cm i.d.) was used. Samples were injected via a 100 µl loop. Absorbances of the eluting fractions were measured using the Pye Unicam detector (see section 3.2.5) and fluorescence was measured using a Perkin-Elmer 204-A fluorescence spectrophotometer (150 W Xenon lamp, band widths, 10 nm). Both spectrophotometers were placed in series with the column (firstly, the UV-Vis spectrophotometer) and were equipped with flow-through quartz cells.

Spectra (uncorrected) of absorbing and fluorescent fractions were recorded at a rate of 60 nm/min after stopping the pump, when a relevant fraction emerged in the spectrophotometer cell.

Fractions containing fluorescent material were fractionated by hand, lyophilized or dried azeotropically at 60^{o}C with dichloromethane under a stream of nitrogen.

Columns were cleaned up by elution with 25 ml methanol and 25 ml methanol: chloroform (1:1, v/v) and routinely cleaned (once a week) by a mixture of dimethyl sulfoxide: methanol (1:10, v/v).

The eluants were degassed by sonication before use, continuously mixed and warmed by a magnetic stirrer during chromatography.

Short theory of liquid chromatography

The retention time of a component on a column can be expressed as elution time, elution volume (V_e), reduced elution volume ($=V_e-V_o$) and R_F ($=V_o/V_e$). In High Pressure Liquid Chromatography (HPLC), the capacity factor (ratio) k' is mainly used:

$$k' = \frac{V_e - V_o}{V_o}$$

where V_o is the column void volume or the elution volume of an unretained sample. V_o has to be determined for each new solvent but most packing materials used in our studies showed no visible shrinking or swelling. Lichrosorb-10RP18 (Merck) is a reversed-phase packing (amorphous silica covalently bound to octadecyl alcohol; mean diameter 10 µm) and is normally developed with polar solvents, which displace first the most polar components.

An increase in solvent polarity gives an increase in sample retention. Two of the main factors which concern the general performance of an HPLC column are selectivity (defined as the capacity of the column to retain different solutes for different times) and the efficiency (the capacity of the column to give narrow solute bands and therefore to effect separation in spite of a low selectivity). The mechanism of retention in reversed-phase chromatography has been a matter of concern. Liquid-liquid partition between the polar mobile phase and a less polar stationary phase, formed by interaction between the eluant

and the bonded phase, has been suggested (Kirkland, 1971). It was also considered that retention is due to a non-polar adsorption (Telepchak, 1973). There seems little doubt that the actual retention mechanism is a complex phenomenon including both of these effects, and is dependent on the type of packing material and other chromatography conditions. For more and detailed information about reversed-phase liquid chromatography the reader is referred to Brown and Krstulovic (1979).

3.2.7. Amino acid analysis

Analysis of isolated HPLC fractions, acid hydrolyzates (6 N HCl, 110°C, 22 h) of yellow proteins and samples of synthetic compounds was performed either on a Rank-Hilger Chromaspek or on a Biotronic LC-6000 amino acid analyzer, applying short and long run conditions, respectively.

3.2.8. Combined gas chromatography- mass spectrometry

GC-MS was performed as described in section 2.2.13, unless stated otherwise.

3.2.9. Gas chromatography

Derivatization of compounds was performed according to methods described in section 2.2.12. In some experiments, compounds were permethylated using diazomethane (CH_2N_2) or persilylated using Tri-Sil/BSA, Formula P, according to methods recommended by the manufacturer (Pierce Chem. Co., Ill.). Flame ionization (FID and nitrogen-FID) was used as detection system.

3.2.10. Pyrolysis-mass spectrometry

Py-MS was performed on yellow protein fractions and reference compounds according to methods to be described in detail in Chapter VII.

Curie-point pyrolysis high resolution mass spectrometry (Py-HR-MS) was performed on a modified MAT 731 double focusing mass spectrometer at the Rijksinstituut voor de Volksgezondheid at Bilthoven, The Netherlands. The apparatus was equipped with a Curie-point pyrolysis reactor fitted to a gas chromatography inlet system (510^{o}C pyrolysis at atmospheric pressure; carrier gas, helium; 70 eV electron impact ionization; ion counting-signal averaging method).

3.2.11. Field desorption mass spectrometry

FD-MS was performed on fractions eluting from the preparative HPLC column at the University of Amsterdam, The Netherlands. A MAT 711 double focusing mass spectrometer equipped with a combined EI, FI and FD source was used. Conditions were: source and temperature 100^{o}C, ionizing voltage 8 kV, emitter temperature (current) 9-12 mA.

Principle of field desorption mass spectrometry (FD-MS)

Using field desorption mass spectrometry, rather non-volatile and high molecular weight compounds can be analyzed. Ionization of the compound is performed at several times $10^{7}Vcm^{-1}$. The sample is placed on the emitter. The mass spectra obtained reveal generally only the molecular ion or an associated

ion composed of the molecular ion and a cation. Samples of ng to µg can be analyzed. For detailed information the reader is referred to reviews by Schulten (1977) and Nibbering (1976).

3.2.12. Thin layer liquid chromatography

For analytical purposes, thin layer liquid chromatography (TLC) plates composed of silica-60 without fluorescent additives were used (plate dimensions, 20x20x0.2 cm). Freshly prepared eluants, composed of n-butanol-acetic acid-water (5:2:3, v/v/v) were used. Staining was performed using ninhydrin spraying agent (Merck, Federal Republic of Germany), after which the plates were heated during 15 min at 50°C. Fluorescent staining of tryptophan and related compounds was performed using a method described by Nakamura and Pisano (1978), utilizing fluorescamine (Fluka, Switzerland) followed by 40% (v/v) perchloric acid.

3.2.13. Dodecyl sulfate gel electrophoresis

SDS gel electrophoresis was performed according to methods described in section 2.2.17.

3.2.14. Peptide mapping

Enzymatic hydrolysis of yellow protein fractions and peptide mapping was performed according to methods described by van der Ouderaa et al. (1973). Only peptides soluble at pH 6.5 were taken into account.

3.2.15. Preparation of reference compounds

Amino acids and tryptophan metabolites were purchased from Sigma London Chem. Co. and used without further purification.

Anthranilic acid (o-amino benzoic acid) was obtained from Merck and recrystallized from hot ethanol.

N-formyl anthranilic acid. The preparation was carried out according to methods described by Dalgliesh (1952) with minor modifications. Acetic acid anhydride (0.25 ml) was mixed with formic acid (98-100%, 0.5 ml) and left on the bench for 30 min, 0.15 ml of this mixture was then added to a solution of anthranilic acid (0.1 g) in formic acid (0.225 ml). After 2 h, a white precipitate was spun down and dissolved in 50% (v/v) ethanol. After cooling to -10°C overnight, crystals formed. These were washed in 50% ethanol and dried *in vacuo* over NaOH pellets. After recrystallization from anhydrous ethyl acetate, the compound had a melting point of 167°C, which is in accordance with the results of Zentmyer and Wagner (1949).

N_1-formyl kynurenine was prepared by the method of Dalgliesh (1952) without modification.

Bityrosine. 3,3'-bityrosine was prepared according to methods described by Gross and Sizer (1959). A 0.1 g sample of L-tyrosine was dissolved in 91 ml water and 5 ml 0.1% hydrogen peroxide (Merck) were added, followed by 4 mg horseradish peroxidase (Sigma type II, 185 purpurogallin units per mg) dissolved in 4 ml water. The pH was adjusted to 9.2 with 6 N NaOH and the mixture was incubated for 16 h at 37°C. A brown pigment formed, which was filtered off. The filtrate was slightly acidified and

lyophilized. The dry material was taken up in water (pH 7.0) and eluted from a Bio-Gel P-2 column (1.6x70 cm). Fractions which were fluorescent at alkaline pH were taken and lyophilized.

Conversion of tryptophan to norharman. Norharman was synthesized according to methods described by Hess and Udenfriend (1959). Tryptophan (50 mg) in 30 ml 0.1 N H_2SO_4 was heated in a boiling water bath with 1 ml 18% (w/v) formaldehyde solution, following which 1 ml 5% hydrogen peroxide was added (dehydrogenation step) and the heating continued for 20 min. After cooling the solution to room temperature, components were isolated on Bio-Gel P-2. No attempt was made to identify individual components in this preparation.

3.2.16 Sugar analysis

Yellow protein samples (2 and 4 mg) were taken in order to determine their sugar content. Neutral sugars and hexosamines were released with 2N HCl (0.2 ml/mg material) during 2.5 h at 100°C in sealed tubes. Fucose, mannose, galactose and glucose were determined after acetylation by means of gas chromatography at 193°C with a 3% ECNSS-M column (Sawardekar et al., 1965) according to methods described by Langeveld et al. (1978).

Of all biochemical reactions, cross-linkage is one by which the smallest amount of interference can produce the greatest amount of damage, to quote Bjorksten (1974). Inasmuch as components for these reactions are plentifully present in all living cells and time available for the reactions to take place is from 70 to 110 years in man, it is certain that these reactions will take place with obvious and unavoidable consequences.

Cross-linkage as a principal causative reaction in aging has been reviewed by Bjorksten (1971)), its nutritional and medical consequences are discussed by several authors (for review, cf. Friedman, 1977) and, in particular, concerning elastin by Guay and Lamy (1979).

Syneretic processes in human lens may occur as a consequence of cross-linking of lens proteins (Bettelheim, 1979), starting with changes in the local hydration of proteins and proceeding to vacuoles and lake formation, in extreme. Increasing disintegration of cell membranes also plays an important role in the formation of the so-called free space in old cells, this free space represents the room in a tissue to which external liquid can penetrate or in which unbound water is gathered. The hard consistence of the inner part of nuclear-cataractous human lenses makes one feel inclined to think that water is lost from this part of the lens, however, determining the water content in lenses with increasing nuclear color and hardness, it was shown that this part of the lens contained as much water as the

soft, transparent cortex (Kramps, 1977). In nuclear-cataractous lenses there seems to be a change in free space too as shown by electron microscopy (Kobayashi and Suzuki, 1975).

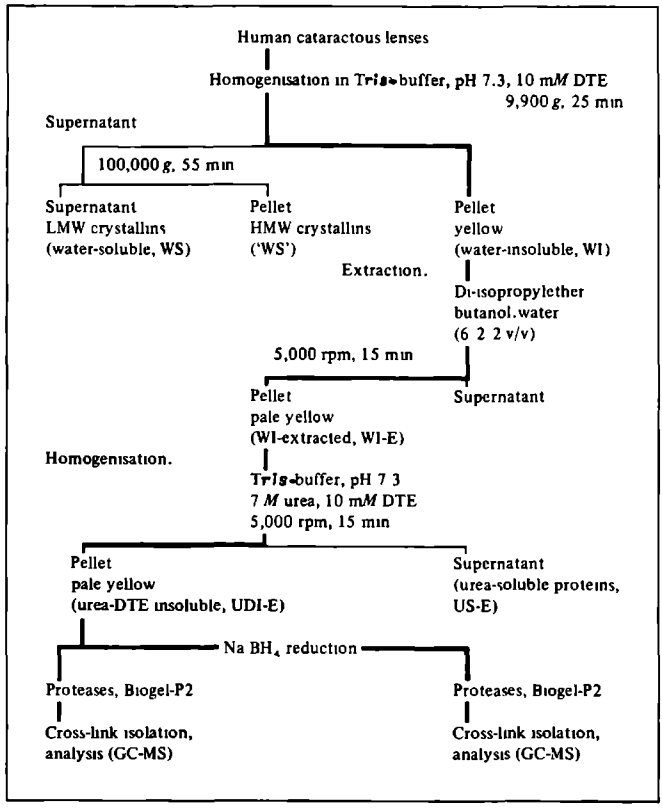
Although acid hydrolysis is the most useful method for quantitatively converting a protein into its constituent amino acids for determination, it has some disadvantages. One of these, the decomposition of tryptophan, has been overcome by replacing the traditionally used 6 N HCl by 3 M p-toluenesulphonic acid or methane sulphonic acid containing tryptamine as an oxygen scavenger (Liu and Chang, 1971). Another disadvantage, the conversion of asparagine and glutamine to aspartic acid and glutamic acid, will always prevent the determination of these individual amino acids after acid hydrolysis. Isopeptide (pseudo-peptide) cross-links will be destroyed and thus escape detection. Chemical modification of proteins cannot always be followed by amino acid analysis using acid hydrolysis due to reversion of modified amino acid residues.

Attempting to isolate unknown compounds including cross-links from the yellow proteins, which turned out to be insoluble in dissociating media like urea/DTE, guanidine-HCl/DTE, SDS and other detergents, we were forced to make a choice between several techniques and their implications. Some of the disadvantages of acid hydrolysis can be overcome by using enzymic hydrolysis. The main advantage of enzymic hydrolysis is that it may release amino acids or amino acid derivatives intactly; disadvantageous may be that a) not all amino acids are released quantitatively due to the specificity of the enzymes

used and b) enzymic hydrolysis is more complicated, time consuming, sometimes very expensive and care must be taken to prevent bacterial growth. What may be a disadvantage in normal protein analysis practice is an advantage in experiments attempting to isolate cross-links: enzymes do not readily split bonds near cross-links, mainly due to steric hindrance. In fact, proteolytic enzymes are designed by nature to hydrolyze proteins containing quite a small range of "natural" amino acid residues. Considering a recent review (Uy and Wold, 1977) stating that over 140 "unnatural" amino acid derivatives have been discovered in proteins derived from living tissue, it is easy to figure which problems may arise in identifying unknown compounds in human lens yellow proteins. More information about the applications and implications of immobilized enzymes used in our study, is given by Everse et al. (1979). It is not always necessary to cleave disulfide bonds in proteins before carrying out enzymic hydrolysis (Milligan and Holt, 1977). New problems arise when studying old lens proteins which may contain racemized amino acid residues (see Chapter IX).

When equal amounts of delipidated water-insoluble fractions from normal and nuclear-cataractous human lenses were digested conform the methods described for the yellow protein fraction, spectral analysis of the solubilized material revealed an increase in the relative abundance of non-tryptophan fluorescent material (excitation wavelength 340, emission wavelengths >435 nm) in relation to the cataractous state (see Chapter II, Fig. 2.12). It was also shown (Chapter II) that the absorbance spec-

Scheme 3.1.



Taken from: van Haard et al. (1978)

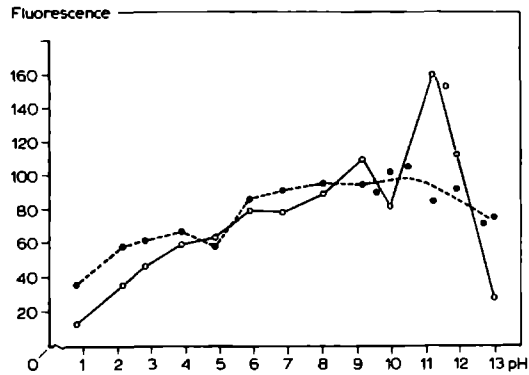


Fig. 3.1. pH-dependence of fluorescence exhibited by digests of the yellow protein fraction from nuclear cataractous human lenses.
Fluorescence intensities are expressed in arbitrary units. Non-tryptophan fluorescence: A/F, 340/410 (●---●); tryptophan fluorescence: A/F, 285/345 (o——o).

trum of digested yellow protein revealed a weak but definite absorption at 340 nm which may be ascribed to fluorophors emitting at wavelengths over roughly 400 nm.

Since the occurrence and the intensity of fluorescence of a compound may be dependent on the pH of the solution (for a review, Duggan et al., 1957), we determined the fluorescence intensity of the digests at several pH values (Fig. 3.1). Non-tryptophan fluorescence (A/F: 340/410) from the digests revealed a weak pH dependence. Fluorescence ascribed to tryptophan and tyrosine (A/F: 285/345) increases at high pH, due to ionization of the phenolic group of tyrosine. Fluorescence intensities at the digestion pH (8.0) and during chromatography (near pH 7.0) do not differ very much as can be concluded from Figure 3.1; at pH below 3 both tryptophan and non-tryptophan fluorescence intensities of the digests seem to be weaker than at high pH. We used the findings, obtained by absorption and fluorescence spectrometry in the following experiments.

In relation to the cataractous state, the elution patterns on Bio-Gel P-2 obtained after digestion of the yellow protein fractions (Fig. 3.2.a) show increasing amounts of material in the void volume of this column; this material cannot be further digested using the enzymes mentioned. For reasons of more realistic depiction we present the transmittance of fractions at 280 nm as recorded by the Uvicord II photometer in Figure 3.2a. As is shown in Figure 3.2b, several fractions eluting from the column show 340 nm absorbance and non-tryptophan fluorescence. Using digestion "buffer" (0.1 M NH_4HCO_3), elution patterns were

Fig. 3.2a *Fig. 3.2b*

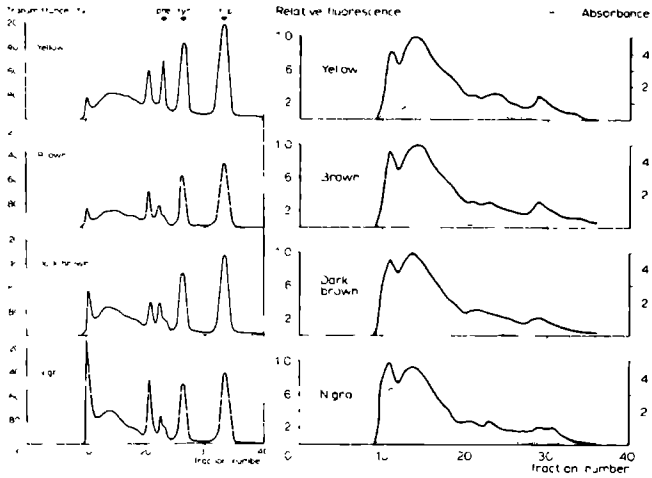


Fig. 3.2a Elution profile of the digest of the yellow protein fraction from nuclear-cataractous lenses. Fractionation was performed on Bio-Gel P-2. Transmittance at 280 nm. For details see Material and Methods.
Fig. 3.2b Relative fluorescence, excitation at 340 nm, emission above 435 nm (——) and 340 nm absorbance (...) of the column effluent shown in Figure 3.2a.

obtained slightly different from those in water (see Chapter II). Aromatic amino acids, e.g. tyrosine and tryptophan, are somewhat retarded on this column, enabling good separation of tryptophan and its more hydrophilic metabolites and also of tryptophan and other amino acids (Table 3.I). As described by Thornhill (1972) compounds with delocalized π-electrons in combination with high polarizability or hydrogen bond formation will be retarded on this column matrix.

Table 3.I *R_F-VALUES OF SOME IMPORTANT AMINO ACIDS ON BIO-GEL P-2*

Amino acid	R_F-value (eluant, 0.1 M NH_4HCO_3, pH 8.0)
Anthranilic acid	2.0-2.1
Phenylalanine	2.1-2.2
Tyrosine	2.4-2.5
Bityrosine	2.4-2.5
Tryptophan	3.1-3.3

Table 3.II *k'-VALUES OF SOME IMPORTANT AMINO ACIDS ON LICHROSORB-10RP18*

Amino acid	k'			
	H_2O	5% MeOH	20% MeOH	20% MeOH, 0.1% Phosph. acid
Anthranilic acid	0	0	1.56	2.47
Tyrosine	2.4	0.66	0	0.04
Phenylalanine	6.4	1.50	0.22	0.47
Bityrosine	-	-	0.28	-
Kynurenine	-	-	0.66	0.27
Tryptophan	-	4.3	1.60	1.81
Norharman	-	>8.0	-	-

Eluant composition is expressed as volume percentage

Several fractions eluting from the P-2 column were analyzed by means of HPLC, using reversed-phase chromatography (Fig. 3.3). Fraction 21 (see Figs. 3.2a and 3.2b) was characterized by a peak revealing absorbance at 260, which eluted in the void volume of the HPLC column. This fraction might represent anthranilic acid. Addition of phosphoric or other acids (Table 3.II) to the eluant resulted in strong retardation

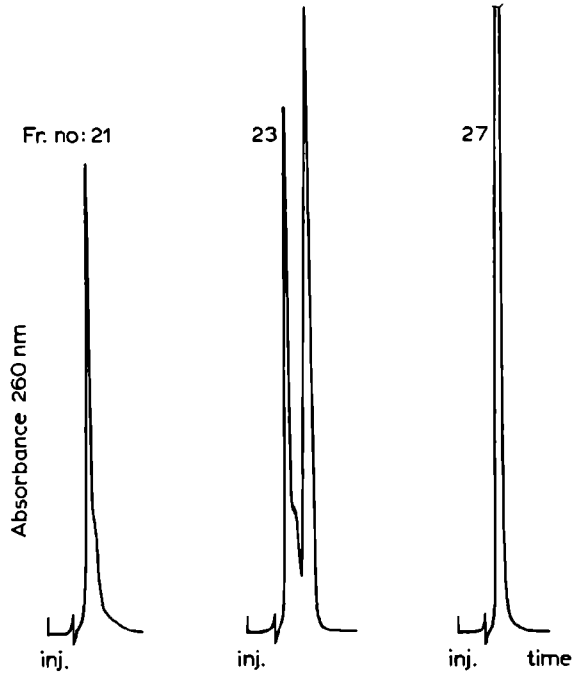


Fig. 3.3. Representative reversed-phase high-pressure liquid chromatograms of fractions eluting from the Bio-Gel P-2 column (see also Figs. 3.2a and 3.2b). Eluant, 5% methanol (v/v), chart speed, 1 cm/min. For more details see Materials and Methods.

of this compound as did authentic anthranilic acid. The fraction eluting from the Bio-Gel P-2 column (fr.no. 21) turned yellow on standing. Fraction number 23 contained, besides the compound mentioned, tyrosine and phenylalanine whereas fraction number 27 contained almost pure tyrosine.

Applying straight-phase TLC and HPLC it was shown that in all low molecular weight fractions eluting from the Bio-Gel P-2 column (fraction numbers 13 and higher non-tryptophan

fluorescent, ninhydrin-negative compounds were present; these compounds were different, by comparison with the relative mobilities and intrinsic fluorescence of authentic material, from bityrosine, anthranilic acid, kynurenine and norharmans and eluted well separated from normal amino acids (R_F below 0.43). Relative mobilities of unknown compounds were higher than that of tryptophan and lower than that of anthranilic acid (Tables 3.III and 3.IV).

Table 3.III *k'-VALUES OF SOME IMPORTANT AMINO ACIDS ON PARTISIL-10*

Amino acid	k' (eluant, n-butanol: acetic acid: water, 5:2:3 (v/v)
Anthranilic acid	0.24
Tyrosine	0.71
Phenylalanine	-
Bityrosine	1.3
Tryptophan	0.59
Norharmans	>6.0

Table 3.IV *R_F-VALUES OF SOME IMPORTANT AMINO ACIDS ON SILICA-60*

Amino acid	R_F-value (eluant, n-butanol: acetic acid: water, 5:2:3 (v/v)
Anthranilic acid	0.79
Tryptophan	0.49
Phenylalanine	0.47
Tyrosine	0.43
Kynurenine	0.45
Bityrosine	0.30

Applying coloring agents according to Nakamura and Pisano (1978), no altered fluorescence was produced, which finding leads to the conclusion that the fluorescent compounds may be indoles like indole-3-acetic acid, but not modified tryptophan-like compounds.

The time elapsed between the HPLC analysis (results depicted in Fig. 3.3) and TLC was rather long, which might be a reason for not finding anthranilic acid on TLC plates. Anthranilic acid used as a reference turned yellow after TLC, as did the fraction from the Bio-Gel P-2 column, which might contain this compound.

Whole digests were applied to TLC plates or straight-phase HPLC columns (Fig. 3.4) immediately after the last digestion step; fast migrating non-tryptophan fluorescent fractions were isolated. Analysis of this material by means of gas chromatography revealed that very small amounts of anthranilic acid were present; authentic anthranilic acid, however, revealed identical retention times as aspartic acid after coinjection, making it impossible to discern these two compounds in a hydrolyzate of the yellow material. No other identifiable and unnatural compounds were present as judged by the GC patterns and using computerized data for GC-MS of amino acids. These findings might be explained by the fact that all non-tryptophan fluorescent compounds spotted on TLC could not be colored with ninhydrin, which fact might be due to lack of oxidizable amino groups (e.g. in the case of anthranilic acid). Fluorescamine was tried but the resulting fluorescence could not be

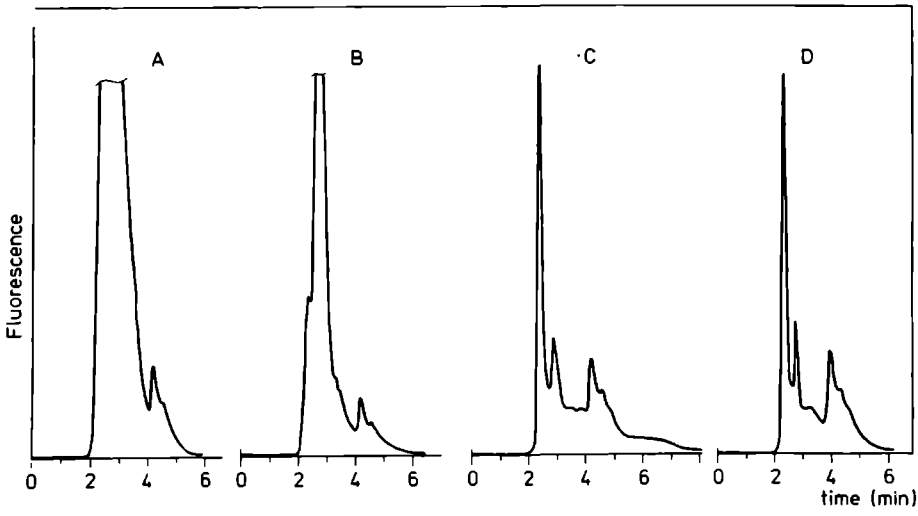


Fig. 3.4. Representative straight-phase high-pressure liquid chromatograms of the digest of the yellow protein fraction from nuclear-cataractous lenses. Eluant, n-butanol: glacial acetic acid: water (5:2:3, v/v/v). Relative fluorescence of effluents is presented: chromatogram A: A/F, 295/345 (tryptophan and indole-fluorescence), B: A/F, 290/410 (fluorescence assigned to bityrosine and substituted anthranilic acid), C: A/F, 335/410 (non-tryptophan-fluorescence), D: A/F, 340 (310 cut-off)/435 (fluorescence assigned to anthranilic acid and kynurenine derivatives). Details are given in Table 3.III and in section 3.2.5.

discerned from the intrinsic fluorescence exhibited by the compounds. The lack of derivatizable amino groups may result in high boiling ester derivatives formed by methods applied (section 2.2.12), which could not be analyzed on the GC columns available. On the other hand, high molecular weight compounds might be present which are fully derivatized (although ninhydrin-negative) but cannot be analyzed on the column type used.

Applying a digest of yellow proteins, derived from 100 nu-

clear-cataractous lenses, to a preparative HPLC reversed-phase column (Fig. 3.5a and 3.5b) revealed the presence of a fast eluting blue fluorescent fraction (at pH 7.0). Chromatograms were

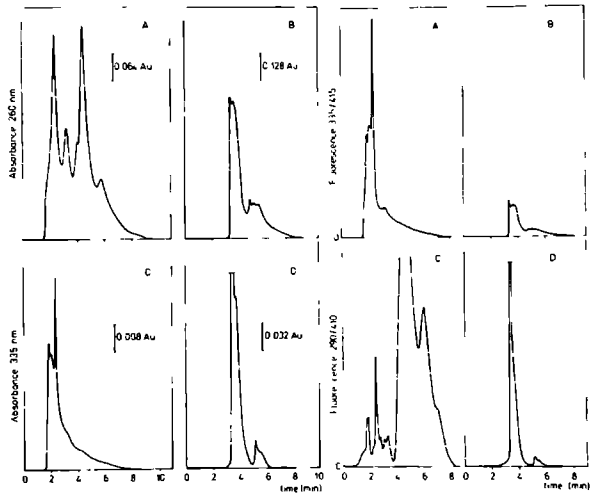


Fig. 3.5. Representative reversed-phase preparative high-pressure liquid chromatograms of the digest of the yellow protein fraction from nuclear-cataractous lenses. Eluant, 20% methanol during 12 min (chromatograms A and C), followed by 100% methanol (chromatograms B and D). Absorbance (3.5a) and fluorescence (3.5b) characteristics are presented in the figures. Fractions eluting shortly after 2 minutes (chromatograms A and C) were taken and extracted with ethyl acetate as described in the text.

reproducible up to 10 injections. After acidification (pH 3.0) and extraction with ethyl acetate, the blue fluorescent compound could be identified as anthranilic acid (o-amino benzoic acid) by means of GC-MS after derivatization using diazomethane or Trisil/BSA-formula P. The mass spectrum and fluorescence

spectrum of anthranilic acid will be shown in Chapter IV, the absorbance spectrum is depicted in Figure 3.6 as well as that

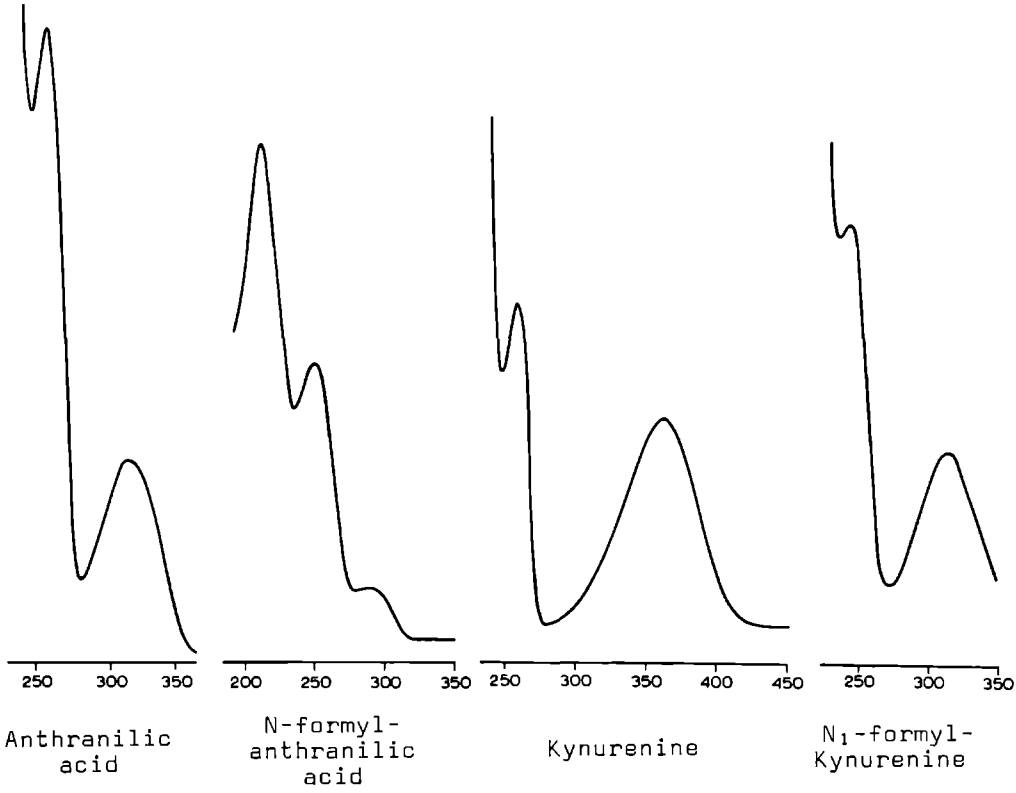


Fig. 3.6. Absorbance spectra of some compounds which may be present in old human lenses. Spectra are corrected for absorbance of the solvent (water, pH 7.0). Concentrations were about 1 mg/ml.

of some compounds which may be present in the human lens. As can be seen, anthranilic acid and both kynurenines (Fig. 3.6) reveal absorbance near 340 nm, making them candidates likely to be present in the yellow protein fraction of nuclear-cataractous lenses.

Many other fluorescent compounds eluted from the HPLC column (Fig. 3.5) some of which turned out to be brown after drying. After cleaning up the column with methanol (Fig. 3.5, chromatograms B and D) an oily and highly fluorescent fraction was isolated, which could not be identified by GC, MS (chemical ionization with methane and electron impact ionization) and revealed a mass peak of 284 by field desorption mass spectrometry. The 90 MHz proton magnetic resonance spectrum of this material in deuterated chloroform (2850 scans, Fourier pulse width 1.6 μsec) revealed the presence of alkanes, which might represent oil out of the HPLC pump and only very small amounts of aromatic compounds, which might represent plasticizers as judged by the field desorption mass spectral data. Proton magnetic resonance (PMR) analysis of other fractions was hampered by insolubility of the compounds to be studied in organic solvents commonly used for PMR analysis.

The yellow protein digests were studied on the amino acid analyzers after loading columns heavily in order to detect small amounts of ninhydrin-positive unknown compounds. The presence of several unnatural amino acids could be seen on the elution patterns; the presence of a compound with identical elution time as γ-amino butyric acid was striking. No bity-

rosine could be found, although being ninhydrin-positive, this compound has been described to be present in cataractous lenses (Garcia-Castineiras et al., 1978a,b) in contrast to the latest results obtained by McNamara and Augusteyn (1980). The latter authors found in accordance with our findings no bityrosine in their yellow protein fraction. Another compound, revealing identical retention time as ornithine was sometimes seen, which finding was in accordance with those obtained by gas chromatography, it was suspected that it was derived from the enzymes used. Anthranilic acid could not be analyzed on the amino acid analyzer since it does not react with ninhydrin. Acid and basic hydrolyzates of yellow proteins revealed no abnormal patterns.

The presence of anthranilic acid in the yellow protein fraction needed further study (see also Chapter IV). After digestion of the yellow proteins with trypsin, followed by peptide mapping (pH 6.5), no anthranilic acid was found. The map did not resemble known maps derived from lens proteins. Very diffuse fluorescent, partly tailing spots were seen all over the maps. Chymotryptic mapping revealed the presence of small amounts of anthranilic acid. After digestion of yellow protein samples according to methods described and extraction with ethyl acetate, about 10 µg anthranilic acid/10 mg could be found in the digests and not in the untreated material, it is assumed that it is covalently bound to lens proteins via pseudopeptide bonds, which are cleavable by some of the enzymes used. Truscott and Augusteyn (1977a) found that anthranilic acid

could be specifically released from the yellow protein by treatment with chymotrypsin. We cannot subscribe such a specificity but trypsin released definitely no anthranilic acid. Other enzymes, especially the proteases obtained from *Streptomyces griseus* might reveal some chymotryptic activity.

Summarizing, yellow proteins from nuclear-cataractous lenses are very difficult to study due to their complex structure as a result of cross-linking (by disulfide and non-disulfide bonds) and other post-translational events.

The presence of anthranilic acid in digests of the yellow protein fractions is indicated in this study. Indole and kynurenine derivatives may also be present, the structure of which remains to be elucidated.

Applying gas chromatography, virtually no sugars could be found to be present in the yellow protein fraction from cataractous lenses varying in nuclear color. No relationship between sugar content and cataractous color state could be found. It seems unlikely that sugars are involved in the process of nuclear browning occurring in nuclear-cataractous lenses.

Although more details will be given about methods used and result obtained in Chapter VII, we like to present in this chapter the results of investigations using pyrolysis high resolution mass spectrometry applied to a normal and a nuclear-cataractous human lens. Ion-signals derived from pyrolysis fragments of lens material are firstly assigned to compounds assumed to be present in the lens material or to be derived

thereoff; pyrolysis high resolution mass spectrometry is then used to provide evidence that the fragments are truely the ones generated by pyrolysis. An ion signal assigned to a mass fragment of 64*was thought to be derived from sulfur dioxide, the latter compound might be produced from methionine sulfoxide present in the parent lens material. As is shown in Fig. 3.7,

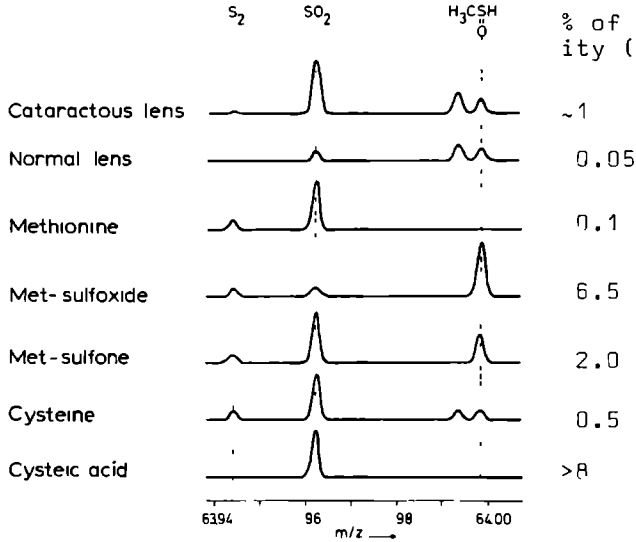


Fig. 3.7. High resolution signals (%) derived from pyrolysis fragments generated by Curie-Point pyrolysis of normal and nuclear-cataractous lenses and reference compounds. *Average mass units are meant.

the exact mass of sulfur dioxide turned out to be 63.961 and is found in normal and nuclear-cataractous human lenses. If we assume that the peak intensities at m/z 64 for the various samples, as obtained by Py-high resolution-MS (MAT 731, modified) are in essence comparable with those obtained by Py-low

resolution-MS (F.O.M. automate, see Chapter VII), it can be concluded that the increase in relative intensity at m/z 64 in the cataractous lens is due to the formation of SO_2. This fragment is derived mainly from cysteic acid, which compound generates a relatively high m/z 64 peak, composed of SO_2 mainly. A contribution from methionine sulfone and sulfoxide might be expected, although in this case an increase in the intensity due to CH_3SOH should occur. It should be mentioned that the relative formation of the pyrolysis products for monomeric model compounds can be somewhat different from that of covalently-linked analogues in eye lens proteins, including oligopeptides such as glutathione, as a result of the influences exhibited by peptide bonds and by matrix effects.

CHAPTER IV

HUMAN LENS NUCLEAR CATARACT : HYDROGEN PERIOXIDE ACTION AND ANTHRANILIC ACID ASSOCIATION WITH LENS PROTEINS 146

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4. HUMAN LENS NUCLEAR CATARACT: HYDROGEN PEROXIDE ACTION AND ANTHRANILIC ACID ASSOCIATION WITH LENS PROTEINS

4.1. Introduction

In the preceding chapter, we described methods for the isolation and characterization of non-disulfide cross-links accumulating in human nuclear-cataractous lenses. The presence of anthranilic acid in digests of the yellow protein fraction was described: this compound was present in very small amounts and was prone to degeneration if not processed immediately. In this chapter we investigated whether European lenses contained anthranilic acid and compared the findings with those obtained from material derived from a large number of Pakistani lenses. We investigated how anthranilic acid might be produced in order to find out from which natural precursors, present in the lens, it might be derived.

4.2. MATERIALS AND METHODS

4.2.1. Yellow protein

Yellow protein fractions were prepared according to methods described in Chapter III (section 3.2.). Material obtained from Pakistan was a generous gift from Dr. K.J. Dilley and Dr. J.J. Harding (Oxford University, Great Britain) who obtained it from Drs. N.E. Christy and P. Laal (Taxilla, Pakistan). In the latter set up, solid guanidine chloride was sent to Pakistan, together with a deionizing cartridge and instruction for making up a 6 M solution. Cataractous lenses (ungraded) were totally immersed in this solution immediately after extraction. Several thousand lenses were collected in this way and sent to the United Kingdom. It was estimated that 2.5 to 3 thousand lenses were received. Brown nuclei were frozen dried, ground in a mortar and pestle and then dialyzed against water at 4^{o}C for 48 h to remove the guanidine chloride. The resulting suspension was then frozen dried and ground to a fine powder to give 60 g of material. About 5 g of this material arrived in Nijmegen and was treated as though it represented the water-insoluble fraction (see section 3.3.2).

4.2.2. Isolation of anthranilic acid

Yellow protein fractions from Europe and Pakistan were digested according to methods described in section 3.3.2. Immediately after digestion and acidification with HCl (pH 3.0), anthranilic acid was extracted twice with 5 ml ethyl ace-

tate. After centrifugation (3,000 g, 15 min), the organic layers were combined and concentrated under a stream of dry nitrogen. The residue was taken up into the eluant used for HPLC (n-butanol-acetic acid-water, 5:2:3, v/v/v). Ten µl samples were applied to a Partisil-10 straight phase column (25x0.46 cm i.d.). Elution was carried out at a flow rate of 2 ml/min (average back pressure, 20 MPa).

4.2.3. Gas chromatography and (pyrolysis) mass spectrometry

Combined GC-MS was performed as described in the text. Pyrolysis mass spectrometry was performed as described in Chapter VII.

4.2.4. Generation of anthranilic acid

In order to find out from which natural precursors, present in the lens, anthranilic acid might be derived, non-deaerated aqueous solutions of tryptophan, the most likely candidate, were treated with dihydroxy fumaric acid according to Goldber and Stern (1977), ferric ammonium sulfate-ascorbic acid according to McCord and Day (1978), hydrogen peroxide according to McCormick et al. (1976) and with near-UV-light (Blak-Ray, long-wave UV-light) according to methods described by Dilley (1973), with only slight modifications. After 40 h at 37°C under each condition, solutions were extracted with ethyl acetate and concentrated organic layers applied to HPLC columns (see section 4.2.2.) or to TLC (Si-60, plate dimensions: 20x20x0.2 cm).

After digestion of yellow protein derived from European and Pakistani lenses, anthranilic acid turned out to be present in about 50 times higher amounts in Pakistani than in European lenses (Fig. 4.1.). Anthranilic acid was identified

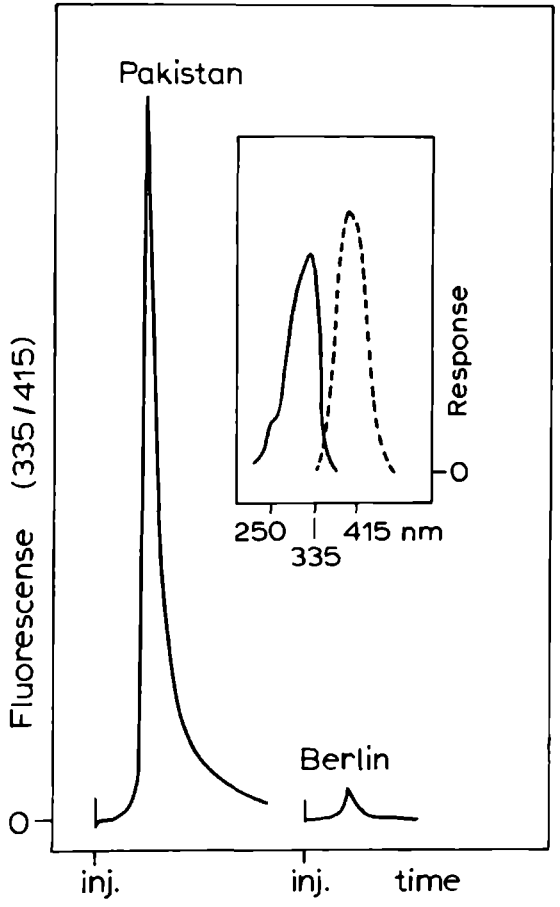

Fig. 4.1. Elution profile on Partisil-10 of the ethyl acetate extracts derived from acidified digests of European (Berlin) and Pakistani yellow protein fractions. The eluant used was n-butanol: acetic acid: water (5:2:3, v/v/v), detection was performed using an excitation wavelength of 335 and an emission wavelength of 415 nm (band widths: 10 nm) determined under stopped-flow conditions (see inset).

by its excitation-emission spectrum (see inset Fig. 4.1.) and by gas chromatography (derivatization to methyl-ester using diazomethane, 100°C, 15 min) in combination with mass spectrometry (LKB 9000, 20 eV, m/z: 92 (10%), 119 (78%), 151 (100%, $m^{+\cdot}$). Mass spectra of the methylesters of isolated and standard anthranilic acid are depicted in Figure 4.2.

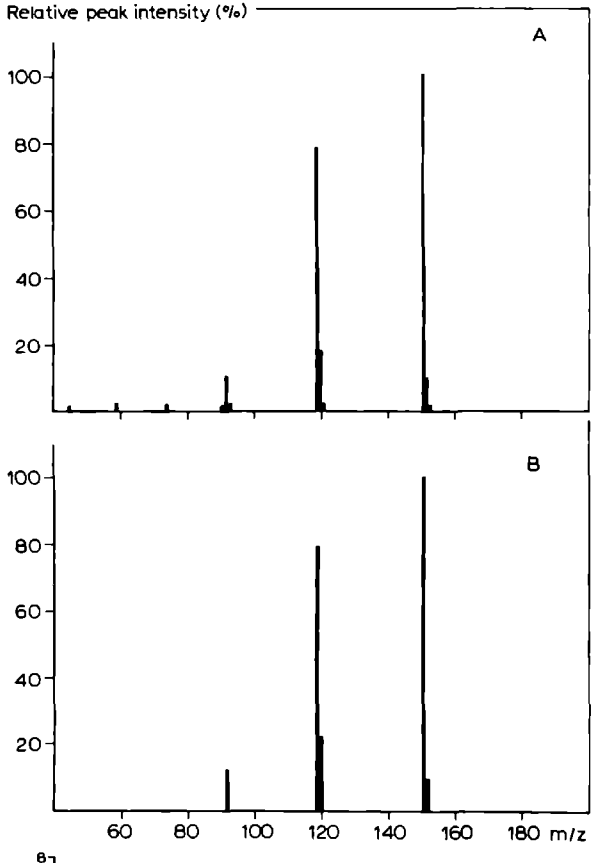

Fig. 4.2. Mass spectra of methylesters of anthranilic acid derived from yellow protein digests (A) and of authentic anthranilic acid (B).

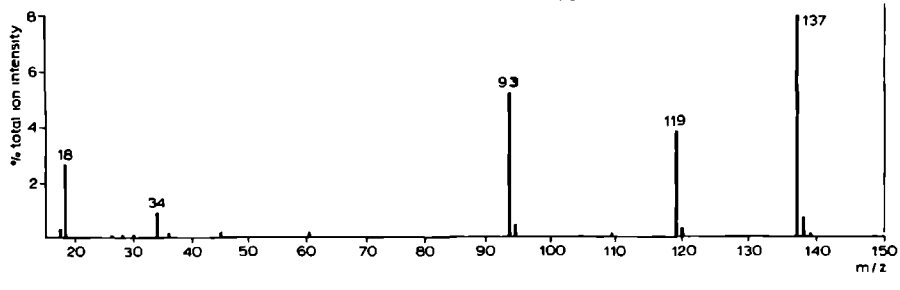

Fig. 4.3. Pyrolysis mass-spectrum of authentic anthranilic acid

Only in the presence of hydrogen peroxide, tryptophan was converted to anthranilic acid amongst others. Fluorescence quenching of tryptophan and other indoles has been noted recently (Omran, 1977; Cavatorta et al., 1979). Hydrogen peroxide, being a good electron scavenger (Hickel and Schmidt, 1970) is able to withdraw an electron from excited indoles. In lens, depending on the physiological environment and the amount of UV-light absorbed, hydrogen peroxide action may lead to the destruction of tryptophan resulting in small amounts of anthranilic acid. It is interesting to note that hydrogen peroxide absorbs light at 300 nm, which is near the threshold wavelength of UV-light penetrating the lens (Pitts et al., 1977) and that it can be produced by near-UV-light action on tryptophan (McCormick et al., 1976).

The Py-MS spectrum of anthranilic acid (Fig. 4.3) shows intensive peaks at m/z 137, 119 and 93. This indicates that this rather volatile compound can be detected in the form of the $M^{+\cdot}$ ion; the fragments at m/z 119 and m/z 93 probably represent $M-H_2O$ and $M-CO_2$ fragments. Inspection of Py-MS spectra of a number of yellow protein preparations showed in some cases the presence of a correlated set of peaks at m/z 135, 119 and 93 in high relative intensities. This set of peaks might be indicative of covalently-linked anthranilic acid residues, however about the type of linkage no definite conclusion could be drawn.

Py-MS spectra of European and Pakistani yellow proteins prepared by different ways (not shown) were highly similar, but no

conclusion could be drawn about the presence of anthranilic acid; essentially no anthranilic acid could be extracted from untreated European and Pakistani yellow protein. Therefore, anthranilic acid might be covalently bound to the yellow protein via a bond which is susceptible to chymotryptic but not to tryptic digestion (see Chapter III). Since chymotrypsin is able to hydrolyse ester bonds between the hydroxy group of tyrosine and for instance acetic acid (Hess, 1971), it may be argued that anthranilic acid becomes bound to tyrosine *in vivo* bond between the carboxylic acid moiety and the hydroxyl group.

Preliminary experiments to determine the anthranilic acid binding capacity of lens proteins and the specificity of this binding, using equilibrium dialysis (see for experimental details Hendriks, van Haard, Klompmakers, Daemen and Bonting, 1977) revealed that almost no binding (less than 2%) occurred at concentrations of anthranilic acid between 20 and 800 µM in 20 mM Tris-HCl buffer (pH 7.3), containing between 0 and 200 mM NaCl. Recoveries were bad (less than 90%) which might be due to adsorption to the dialysis bags (Visking tube), glass tubes and to light or oxidation. No anthranilic acid was released enzymatically from individual lens proteins incubated with anthranilic acid, which were separated by means of gel chromatography on Bio-Gel A-5m. The question remains therefore how anthranilic acid becomes bound to the yellow protein fraction if not through a pathway of generation from an unbound precursor, followed by covalent attachment to lens proteins by a bond susceptible to proteolytic digestion.

SULFHYDRYL GROUPS IN INDIVIDUAL NORMAL AND NUCLEAR-CATARACTOUS HUMAN EYE LENSES

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Sulfhydryl Groups in Individual Normal and Nuclear-Cataractous Human Eye Lenses

A Study Emphasizing Age, Cataractous State and Cataract Localization

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Key Words. Human lens Aging Nuclear cataract Sulfhydryl content Nucleus Cortex Water-soluble fraction Urea-soluble protein

Abstract. Evaluation of sulfhydryl data derived from the nucleus and cortex of individual human lenses, ranging in age from 40 to 90 years, revealed the following (a) no significant correlation between the age and the sulfhydryl content of normal and nuclear-cataractous lenses, (b) a significant relationship between the distribution of non-protein-bound sulfhydryl compounds among the lens parts and both age and cataractous state, (c) no significant difference in sulfhydryl data from the cortex between groups of cataractous lenses with increasing nuclear color, and (d) a significant difference in nuclear protein sulfhydryl content between the groups of lenses, varying in cataractous state If lens parts are separated on the basis of cataract localization, sulfhydryl oxidation in nuclear cataractogenesis seems to be confined to the colored nuclear region It is suggested that nuclear cataract develops from the inner nucleus towards the cortex of the lens

Introduction

Nuclear-cataractous human eye lenses have been divided into four groups according to increasing nuclear color based on *in vivo* and *in vitro* examination of the intact lens [*Kramps et al.*, 1976]. Biochemical findings in our laboratory [*Kramps et al.*, 1976; 1978] indicated a definite relationship between the four groups as classified by the ophthalmologist. Chiefly the nuclear part of this type of cataractous lenses contains proteins that become progressively polymerized, oxidized, fluorescent and colored; these proteins contain also increasing amounts of age-dependently degraded polypeptide chains and racemized amino acid residues [*Kramps et al.*, 1976, 1978; *Bando et al.*, 1976; *Takemoto and Azari*, 1977; *Trus-*

cott and Augusteyn, 1977a–c; *Masters et al.,* 1978; *Garner and Spector,* 1978; *Roy and Spector,* 1978; *Garcia-Castineiras et al.,* 1978]. It is so far an open question why only the nucleus of these lenses changes color and morphology without losing water, leaving the cortex relatively unchanged. The most accepted theory about the development of nuclear cataract is based on the mere fact that part of the ultraviolet light emitted by the sun (> 295 nm) can enter the lens through the cornea [*Bachem,* 1956; *Pitts et al.,* 1977]. It has been shown to be impossible to induce changes limited to the nucleus of intact human lenses [*Zigman,* 1977; *Lerman et al.,* 1976a, b]. Raman spectroscopic measurements on intact lenses [*East et al.,* 1978] seem to be promising in finding nuclear alterations. It has been postulated that UV light induces changes in lens proteins via free radical mechanisms [*Borkman and Lerman,* 1977; *Yamanashi and Zuclich,* 1978; *Weiter and Subramanian,* 1978]. Finding a lack of radical scavengers like glutathione and ascorbic acid, accumulation of damaged components and decreasing enzyme activities in the nuclear part of the lens might substantiate such a hypothesis. The role of sulfhydryl groups in the maintenance of lens transparency has been widely studied [for a review, cf. *Harding and Dilley,* 1976].

In order to clarify the role of sulfhydryl groups in senile cataract formation, *Truscott and Augusteyn* [1977d] and *Anderson and Spector* [1978] separated human lens cortices and nuclei by means of cork borers with constant internal diameter (5 and 6 mm, respectively). After slicing off the ends of the cylinders obtained, these investigators determined the changes in sulfhydryl contents depending on the state of cataract.

In our opinion methods like this are not completely adequate in studying changes in nuclear-cataractous lenses. In our laboratory and by others [*Bando et al.,* 1976] the noncataractous cortex is separated from the colored nucleus by peeling decapsulated lenses until the hard nucleus is obtained.

In a systematic study, we investigated individual normal and nuclear cataractous human lens sulfhydryl levels at progressive stages of cataract, separating nuclei and cortices on the basis of cataract localization.

Materials and Methods

Lenses. Nuclear-cataractous lenses were obtained from operations at the Eye Clinic of the Free University of Berlin. The lenses were classified *in vivo* and *in vitro* into four groups of increasing nuclear color: lenses with a yellow nucleus (Ye), a brown nucleus (Br), a dark

brown nucleus (Db) and a black nucleus (Nı cataracta nigra) After careful examination lenses were stored in liquid nitrogen Normal (No) lenses were obtained within 5 h after death of individuals, 40–90 years of age from the Eye Hospital at Rotterdam The Netherlands

Preparation of Lens Parts Lenses were slowly thawed on an ice-cooled glass plate covered with parafilm After incision of the capsule at the anterior side, the cortices were removed with a spatula immobilizing the hard nuclei by means of a needle This procedure took no more than 3 min/lens Wet weight and water content of this type of cataractous lenses are unchanged [*Kramps et al*, 1976] Normal lenses were treated the same way, considering the inner 30–40% of the lens wet weight to be the nucleus Isolated lens parts were kept on ice under nitrogen atmosphere to avoid oxidation by air

Determination of Sulfhydryl Groups Each cortex (Co) and nucleus (Nu) was homogenized and processed further essentially according to the methods described by *Truscott and Augusteyn* [1977d], determinations of non-protein-bound sulfhydryl (NP-SH) free protein sulfhydryl (P-SH) and protein bound sulfhydryl (PB-SH) being modifications of methods described by *Sedlak and Lindsay* [1968] Freshly prepared, degassed urea solutions were kept in contact with mixed bed ion exchanger until used

Protein Determination Protein was determined according to *Lowry et al* [1951] after incubating samples overnight with 0 1 *N* NaOH, 2% (w v) Na_2CO_3 Protein contents were calculated by reference to standard curves using bovine serum albumin or calf α-crystallin (0–200 μg)

Statistical Evaluation An F test was applied in order to test the hypothesis that, at a level of significance of 0 05, no linear relationship exists between the values of sulfhydryl data within each group of lenses and lens age

If this hypothesis was not rejected one-way analysis of variance ($\alpha = 0\,05$) was applied, neglecting lens age in order to test whether the results from lenses, varying in cataractous state, were different When these differences were significant we determined which lens group data were different from other group data using the simultaneous method of *Scheffe* [1959]

If the first hypothesis was rejected, lenses were divided into two age groups (≤ 65 and > 65 years) one-way variance analysis was applied in order to test, within each age group, whether the results from lenses, varying in cataractous state were different

Results and Discussion

Table I lists relevant data obtained from the non-protein-bound sulfhydryl compounds (NP-SH) in cortices and nuclei of 48 normal and nuclear-cataractous human lenses. Duplicate determinations were performed on each lens part (mean values were used for evaluation) Wide variations were observed in the absolute values of the NP-SH compounds (expressed per lens part), of which glutathione represents the major compound (>90%) as judged from amino acid analysis Negligible Folin-positive material was found in the TCA/EDTA supernatants In all groups of

Table I. Nuclear and cortical non-protein-bound sulfhydryl groups (NP-SH) of individual normal and nuclear-cataractous human lenses[1]

Nuclear color	Age, years	n	NP-SH, nmol		NP-SH/Nu, % of total
			Nu	Co	
Normal	61 8 ± 12.2	10	67 7 ± 40 2	212 7 ± 145.1	25.2 ± 6 1
Yellow	65.8 ± 14 6	14	65 0 ± 48 0	207 4 ± 120 7	22.4 ± 8 4
Brown	70 8 ± 8.4	8	58 5 ± 41 4	213 9 ± 141 7	21 1 ± 7 9
Dark brown	67 4 ± 9.6	8	36 4 ± 26 6	206 6 ± 136 2	15 3 ± 7 8
Nigra	68 3 ± 19.0	8	32.9 ± 25.9	276 0 ± 161 2	11 5 ± 3 9

[1] Data are expressed as mean value ± SD Statistical evaluation is given in the text. n = Number of observations, Nu = nucleus, Co = cortex

lenses examined, the cortex contained significantly more NP-SH than the nucleus of the same lens ($p < 0.05$; two-tailed paired t test). Moreover, every cortex contained more NP-SH than the corresponding nucleus. These findings are in accordance with those obtained by *Truscott and Augusteyn* [1977 d]. The results obtained lead to the following conclusions:

We could not find a significant correlation between the amounts of NP-SH and the age of normal and nuclear-cataractous lenses, determining NP-SH in separated cortex (F 1.607; d.f. 5,38; p 0.18) and nucleus (F 0.940; d.f. 5,38; p 0.47).

No significant differences in the amounts of both cortical and nuclear NP-SH were found (F 0.38; d.f. 4,43; p 0.82 and F 1.59; d.f. 4,43; p 0.19, respectively) between groups varying in cataractous state. To confirm the hypothesis that sulfhydryl groups are involved in cataractogenesis, changes in the NP-SH content should be found in the cataractous nucleus. If the proportion of total NP-SH groups as found in the nucleus (NP-SH/Nu) of each lens is calculated (table I) a significant correlation is found between this proportion and lens age (F 2.74; d.f. 5,38; p 0.03). Dividing lenses into two age groups (table II) reveals that in both age groups a significant difference exists in this proportion (NP-SH/Nu) between the lenses varying in cataractous state (age ≤ 65 years: F 3.36; d.f. 4,16; p 0.04; age > 65 years: F. 3.95; d.f. 4,22; p 0.01). In the age group older than 65 years we can make a distinction between the normal and yellow

Table II. Proportion of NP-SH as found in the nucleus from normal and nuclear-cataractous human lenses, divided into age groups

Nuclear color	NP-SH/Nu, % of total	
	≤65 years	>65 years
Normal	27.4 ± 5.9 (7)	20.0 ± 2.0 (3)
Yellow	22.0 ± 10.7 (7)	22.7 ± 6.1 (7)
Brown	31.0 ± 1.4 (2)	17.8 ± 5.9 (6)
Dark brown	24.0 ± 4.2 (2)	12.3 ± 6.4 (6)
Nigra	10.0 ± 2.7 (3)	12.5 ± 4.5 (5)

lenses as opposed to the dark brown and nigra lenses. Using the S-method of *Scheffé* [1959] the yellow lenses, in age higher than 65 years, show a significantly higher proportion of NP-SH/Nu than the dark brown group (F-Scheffé 2.77, d.f. 4,22, p 0.05) and than the nigra group (F-Scheffé 2.47; d.f. 4,22; p 0.07). In the age group 65 years and younger only the nigra group is almost significantly different from the normal group (F-Scheffé 2.74, d.f. 4,16, p 0.06). At first sight our results are conflicting with the conclusions drawn by *Truscott and Augusteyn* [1977d]. We have to conclude that no changes occur in the NP-SH content of the transparent cortices and colored nuclei at any state of nuclear cataractogenesis (although table I reveals a slight decrease in contents of the nucleus) but considering each lens, varying in cataractous state, the proportion of total NP-SH as found in the cataractous part of the lens changes significantly upon cataract development. Moreover, as is elucidated in table III (fifth column), the colored nucleus may represent 30–50% of the wet weight of the lens depending on the state of cataract. This finding leads to the conclusion that, although the absolute amount of NP-SH is not significantly reduced when comparing cataractous nuclei, the distribution of NP-SH within each lens changes. Going from yellow to nigra, the concentration of NP-SH in the nucleus diminishes about threefold during nuclear cataractogenesis. We have calculated this factor, utilizing the data of tables I and III knowing that the wet weight of the lens and the water percentage of the lens parts do not change upon nuclear cataract development.

The decrease in NP-SH concentration could be due to removal of NP-SH groups by mixed disulfide bond formation with nuclear proteins, to

Table III. Nuclear-cataractous and normal human lens data[1] (mean ± SD)

Nuclear color	Age, years	n	Wet weight, mg	Nucleus wet weight[1] %	Equatorial radius, mm	Equatorial width, mm
Normal	69 ± 7	10	234 ± 29	–	9.2 ± 0.4	4.8 ± 0.4
Yellow	72 ± 12	12	213 ± 29	32 ± 6 (3)	9.0 ± 0.3	4.6 ± 0.5
Brown	72 ± 8	10	226 ± 21	40 ± 4 (3)	9.0 ± 0.8	4.8 ± 0.5
Dark brown	73 ± 8	10	211 ± 42	46 ± 8 (3)	8.7 ± 0.4	4.7 ± 0.6
Nigra	79 ± 8	7	229 ± 36	50 ± 2 (3)	9.1 ± 0.4	4.8 ± 0.7

[1] Determinations were performed on number of lenses indicated between parentheses. The lenses used were not different in age from other lenses within the same group.

decreased regenerability of oxidized glutathione or to decreased permeability of glutathione towards the nuclear part of the lens during nuclear cataract development.

The amounts of glutathione bound to the proteins of both cortex and nucleus from normal and nuclear-cataractous lenses were very low in our investigation (these results will not be given here). Extremely low values were found in the cortices of both normal and nuclear-cataractous lenses; wide variations were obtained in nuclear amounts, although we did not always find more protein-bound sulfhydryl groups (PB-SH) in the nucleus than in the corresponding cortex. From these findings it can be concluded that, as far as the water- and urea-soluble proteins is concerned, the decrease in free glutathione concentration cannot be explained by cross-linking between glutathione and protein sulfhydryl groups (less than 0.2%).

As described in a recent publication [*Kramps et al.*, 1978] evidence has been obtained that the urea-soluble lens proteins from nuclear-cataractous human lenses contain no intermolecular disulfide bonds, but intramolecular disulfide bonds and bonds between proteins and low molecular weight compounds (like glutathione or small peptides) could not be denied. To investigate these findings further we determined the free sulfhydryl groups (P-SH) of the water- and urea-soluble proteins from lens parts at different age and state of cataract. In contrast to the NP-SH levels, the free protein sulfhydryl group (P-SH) values for lens parts show less variability (table IV). The conclusions to be drawn from table IV are: (1) no age-related changes in the P-SH content of water- and urea-soluble proteins could be found in both cortex and nucleus of normal and

Table IV. Nuclear and cortical free sulfhydryl groups in water- and urea-soluble proteins (P-SH) of individual normal and nuclear-cataractous human lenses[1]

Nuclear color	Age, years	n	P-SH, mol mol protein[2]	
			Nu	Co
Normal	62 1 ± 12 1	10	1 49 ± 0 31	1 86 ± 0 19
Yellow	65 8 ± 15 1	13	1 33 ± 0 42	1 90 ± 0 39
Brown	70 7 ± 9 8	6	1 03 ± 0 30	1 66 ± 0 45
Dark brown	69 9 ± 15 7	7	0 93 ± 0 24	1 84 ± 0 24
Nigra	68 0 ± 18 9	6	1 02 ± 0 46	1 53 ± 0 60

[1] Data are expressed as mean values ± SD Statistical evaluation is given in the text n = Number of observations, Nu = nucleus, Co = cortex

[2] Calculated assuming an average subunit molecular weight of 20,000 daltons.

nuclear-cataractous lenses (F 1.344; d.f. 5,32; p 0.27 and F 0.937; d.f. 5, 32; p 0.48, respectively); (2) no significant difference was found in the level of cortical P-SH between the groups of lenses, varying in cataractous state (F 1.260; d.f. 4,37; p 0.30); (3) a significant difference in nuclear P-SH was found between the groups of lenses (F 3.59; d.f. 4, 37; p 0.01). We could make a distinction again between normal and yellow lenses as opposed to the brown, dark brown and nigra group. The S-method of *Scheffé* [1959] revealed an almost significantly lower P-SH content of the dark brown lens nuclei than of the normal nuclei (F-Scheffé 2.41; d.f. 4,37; p 0.07). Again our results are conflicting with those obtained by *Truscott and Augusteyn* [1977d], but it might be argued that the decreases in P-SH content in the nuclear region and the unchanging levels in cortical region found in our investigation are likely to be the result of a clear-cut separation of cataractous and noncataractous lens parts. Although more advanced cataracts show a very low nuclear P-SH content, this is not a general phenomenon. Since we found no decrease in cortical P-SH in relation to the cataractous state, any change in protein sulfhydryl groups, if involved in cataractogenesis, should be found in the nuclear region and this assumption has been confirmed in table IV.

A comparison of our findings with those of *Truscott and Augusteyn* [1977d] leads to the conclusion that nuclear proteinfree sulfhydryl levels decrease during nuclear cataractogenesis due to either mixed disulfide

bond formation, predominantly intramolecular bonds, and/or to other oxidation processes, or disappearance of sulfhydryl-containing proteins. Since we isolated nuclei on the basis of cataract localization (growing in size), results from our investigation may lead to the conclusion that nuclear cataract grows from inner nucleus to outer cortex; changes in sulfhydryl content may start at the inner nucleus, which is smaller in size than the newborn lens, spreading out towards the cortex when nuclear cataract develops under the influence of radical attack on proteins and other lens components. The age-dependently changing physiological environment of nuclear proteins could determine the moment at which nuclear cataract starts; as can be seen in table I the amounts and concentration of glutathione even in normal lens nuclei can be low enough to not overcome the radiation attack by UV light from the sun.

From their electronic structure it can be deduced that sulfhydryl groups protect against ionizing radiation [*Nagata and Yamaguchi,* 1978]; in human lens this action becomes that of a two-sided sword leading to altered protein conformation resulting in insoluble aggregates. Next to the nontryptophan fluorescent nondisulfide covalent bonds there must be many intra- and intermolecular disulfide bonds formed in nuclear proteins from this type of cataractous lenses resulting in making them even urea-insoluble. Further experiments have to be done to elucidate the sequence in which disulfide and nondisulfide bond formation takes place and which components play a part, besides sunlight, in these processes occurring at old age.

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CHAPTER VI

TRYPTOPHANYL GROUPS IN INDIVIDUAL NORMAL AND NUCLEAR-CATARACTOUS HUMAN LENSES

6. TRYPTOPHANYL GROUPS IN INDIVIDUAL NORMAL AND NUCLEAR-CATARACTOUS HUMAN LENSES

6.1. Introduction

The most accepted theory about the development of nuclear cataract is based on the mere fact that part of the ultraviolet light emitted by the sun (>295 nm) can enter the lens through the cornea. It has been postulated that UV-light induces changes in lens proteins via free radical mechanisms. Whether these changes occur concomitantly with destruction of tryptophan in lens proteins is discussed in this chapter. It has been reported that irradiation of proteins and tryptophan *in vitro* causes yellowing, cross-linking (polymerization) and protein insolubilization. Whether these events have taken place in the human lens upon nuclear cataractogenesis was investigated in proteins from several parts of the lens, assuming precursor relationships, changes to be found in the water- and urea-soluble fraction of human lens parts might indicate where and when nuclear cataract develops. As far as the proteins are concerned, it is questioned whether their coloration occurs when they are still in the soluble state.

6.2. MATERIALS AND METHODS

6.2.1. Lenses

Human nuclear-cataractous lenses were obtained from operations at the Eye Clinic of the Free University of Berlin (FRG). The lenses were classified *in vivo* by means of a slitlamp into four groups of increasing nuclear color: lenses with a yellow nucleus (Ye), a brown nucleus (Br), a dark brown nucleus (Db) and a black nucleus, ophthalmologically termed: cataracta nigra (Ni). Immediately after surgery and re-examination, lenses were stored in capped vials at -70°. Transport to Nijmegen took place in a container filled with liquid nitrogen. To prevent lyophilization during storage (-80°C), lenses in capped vials were sealed under vacuum in plastic tubings.

Normal, completely transparent lenses were obtained within 5 h after death of individuals at various ages from the Eye Hospital at Rotterdam, The Netherlands.

6.2.2. Preparation of lens parts

Lens parts were isolated as described in Chapter II, section 2.2.3.

6.2.3. Determination of tryptophan

Each lens was homogenized and processed according to methods also used for the determination of sulfhydryl groups (Chapter V): lens parts were homogenized in 1 ml ice-cold 10% (w/v) TCA, 0.02 M EDTA. After centrifugation (3,000 g, 15 min) pellets were washed with 10% TCA and the pooled supernatants

adjusted to 2 ml. The pellets were washed with 3 ml 10% TCA and finally solubilized in 7 M urea, 0.1 M NH_4HCO_3, 0.02 M EDTA, (pH 8.0). After centrifugation (3,000 g, 15 min), in order to remove urea-insoluble material, the urea-soluble fraction was adjusted to 2 ml. Tryptophan was determined according to Neitchev and Boudevska (1975), with minor modifications. Samples from the TCA-supernatants (0.2 ml) and water- and urea-soluble lens fraction (25 µl) were adjusted to 1 ml and 0.1 N HCl. Color reagent was prepared by mixing 0.1% (w/v) fructose, 75% (v/v) sulfuric acid and 2.5% (w/v) cysteine-HCl, in this order, to obtain a volume ratio of 2:9:3 (v/v/v). This mixture was heated during 10 min at 50°C, immediately after heating, 4.85 ml were added to the samples under vigorous mixing. A pink color developed and, after 0.5 h at room temperature, the absorbance at 518 nm was read against an appropriate blank. Bovine serum albumin, ribonuclease A, myoglobin, histon FII, trypsin (TPCK-treated), hemoglobin, cytochrome C, ovalbumin, bovine α-crystallin and lysozyme were used as test samples. Tryptophan contents were calculated by reference to a standard curve using tryptophan (0-20 µg).

6.2.4. Protein determination

Protein was determined according to Lowry et al. (1951), after incubating samples overnight with 0.1 N NaOH, 2% (w/v) Na_2CO_3. Protein concentrations were calculated by reference to a standard curve using bovine serum albumin or calf α-crystallin (0-200 µg).

6.2.5. Statistical evaluation

Hypothesis testing was performed as described in Chapter V.

When tryptophan is treated with the fructose-sulfuric acid-cysteine reagent, a pink chromophore develops, revealing the absorbance spectrum as depicted in Figure 6.1. The calibration curve for the determination of tryptophan, shows good linearity up to 20 µg tryptophan (Figure 6.2.). The tryptophan content of various proteins tested are given in Table 6.I. With the exception of lysozyme, good correlation with previously reported values was obtained. Proteins like ribonuclease A, which lack tryptophan, do not react; also no reaction is observed when testing tyramine, 3-hydroxy-tyramine, kynurenine,whereas 5-hydroxy-tryptophan reacted slower and melatonine reacted at almost the same rate as tryptophan. The indole moiety seems to be necessary for color development.

Non-protein-bound tryptophan (or other indoles) were determined in lens parts of individual normal and nuclear-cataractous lenses. In all lenses studied, the free indole content was less than 1 µg per lens part. This result indicated that indoles were present, although in very low concentration. No statistical evaluation was feasible. Negligible Folin-positive material (<5 µg per lens part) was found in the TCA-supernatants, indicating that proteins were precipitated almost quantitatively.

The tryptophan levels of the water- and urea-soluble protein fraction (P-Trp) from lens parts from lenses of various age and state of cataract fall into a fairly narrow range,

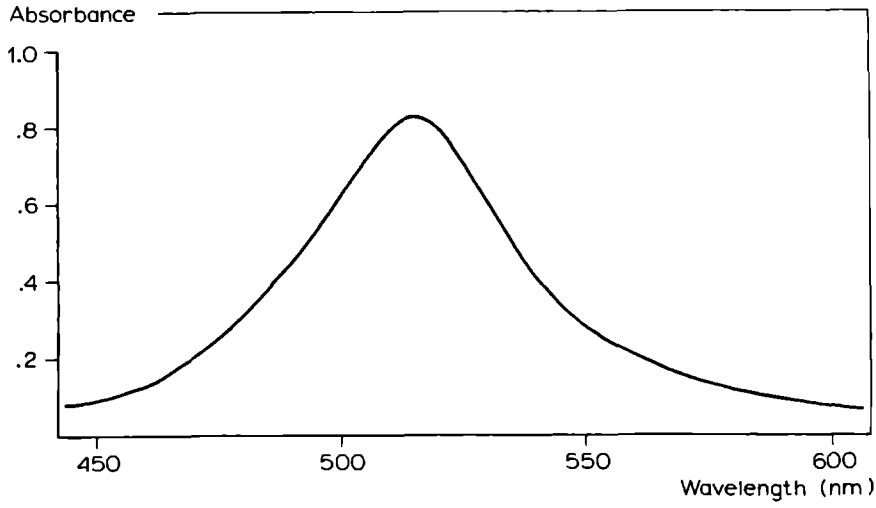

Fig. 6.1. Absorbance spectrum of the chromophore formed by reaction of tryptophan with the fructose-sulfuric acid-cysteine reagent.

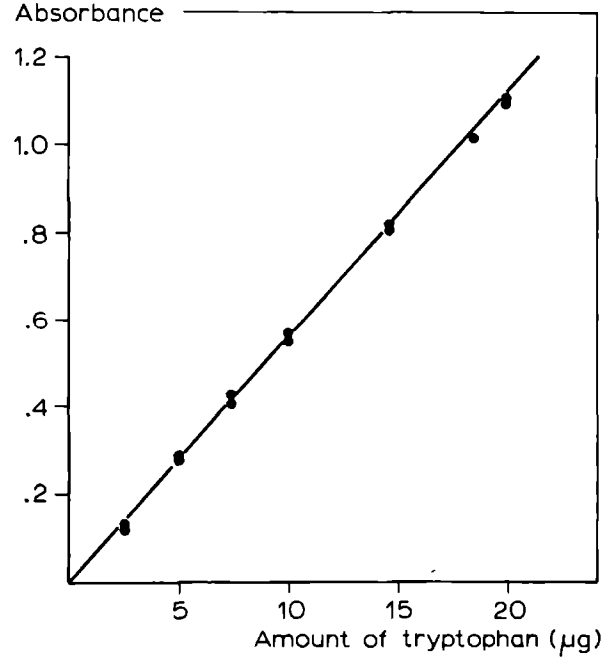

Fig. 6.2. Absorbance of the pink chromophore (518 nm) formed on treatment of different amounts of tryptophan with the fructose-sulfuric acid acid-cysteine reagent.

Table 6.I. THE TRYPTOPHAN CONTENT OF VARIOUS PROTEINS DETERMINED USING THE FRUCTOSE-SULFURIC ACID-CYSTEINE REAGENT AND A COMPARISON OF THE RESULTS WITH PREVIOUSLY REPORTED VALUES.
The number of determinations are given in parentheses; data are expressed as mean ± S.D.

Protein	M_r	Tryptophan content (moles/moles protein) This method[a]	Reported values
Bovine serum albumin	66,000	1.98 ± 0.07 (5)	2.17[b], 2.0[c,d]
Ribonuclease A	12,640	0.01 ± 0.01 (6)	0.06[b], 0.1[f], 0.0[c]
Myoglobin	16,890	1.9 ± 0.5 (4)	1.55[b], 2.0[f]
Histon FII	15,500	0.03 ± 0.00 (5)	0.04[b], 0.0[g]
Trypsin (TPCK-treated)	23,800	4.29 ± 0.10 (4)	3.04[b], 3.2[c], 4.0[d]
Hemoglobin	64,500	5.01 ± 0.70 (6)	5.50[b], 6.0[f]
Cytochrome C	13,400	0.99 ± 0.10 (6)	1.01[b], 0.8[e], 1.0[f]
Ovalbumin	46,000	2.4 ± 0.2 (6)	2.26[b], 2.7[e]
Lysozyme	14,900	2.97 ± 0.01 (3)	5.97[b], 6.0[f]
Bovine α-crystallin	850,000	1.2 (1)	1.25[h]
Bovine α-crystallin A_2	19,500	1.0 (1)	1.00[h]
Bovine α-crystallin B_2	20,070	1.8 (1)	2.00[h]

[a] A protein solution containing 0.2 to 2 mg protein in 25 µl 7 M urea, 0.1 M NH_4HCO_3, pH 8.0 was adjusted to 1 ml and 0.1 N HCl. Color reagent was added and absorbance measured after 0.5 h at 518 nm.

[b] Basha and Roberts (1977)

[c] Sasaki et al. (1975)

[d] Hugli and Moore (1972)

[e] Spande and Witkop (1967)

[f] Dayhoff (1972)

[g] Tristram and Smith (1963)

[h] van der Ouderaa et al. (1973, 1974)

the conclusions to be drawn from Table 6.II are:

- no age-related changes in the P-Trp content of water- and urea-soluble protein could be found in cortex, nucleus and embryonic nucleus from normal and nuclear-cataractous lenses (F-test, α=0.05)
- in all groups of lenses and in each lens no significant difference in the P-Trp content of the water- and urea-soluble fraction of cortices and nuclei could be found ($P>>0.05$; two-tailed paired t-test)
- between groups of lenses, varying in cataractous state, no significant difference was found in the level of cortical P-Trp (F 1.09; d.f. 4,31; $P>0.10$) and nuclear P-Trp (F 2.19; d.f. 4,23; $P>0.10$). Since embryonic nucleus data were derived from another batch of lenses, we compared mean data of cortices and nuclei of the first with mean data from embryonic nuclei of the second batch (Students t-test), no difference in the levels of P-Trp from the first batch of lenses (cortices and nuclei) and the second batch of lenses (embryonic nuclei) was found ($P>0.10$).

Before rejecting the hypothesis that UV-light causes destruction of tryptophanyl groups in and coloration of lens nuclei upon nuclear cataractogenesis, the results obtained may be interpreted in several ways. One of the most important facts about nuclear cataract development is that deepening of nuclear color is accompanied by an increase in "colored nuclear space" (see Chapter V). This observation has led to the hypothesis that nuclear cataract starts at the oldest

part of the lens (the embryonic nucleus) and grows upon maturation, towards the periphery. We did not find changes in the tryptophan levels of the water- and urea-soluble proteins in relation to age or cataractous state. Explanations for this may be: a) damage to lens protein tryptophan, leading to the development of color, might occur only at the stage when nuclear (i.e. old) proteins have become insoluble *in vivo* and experimentally even in urea solvent; b) coloration of "soluble" lens proteins may be confined to only few proteins, occurring in minor amounts, which are also involved in the protein insolubilization process, which is amongst others due to disulfide bond formation; the changes in the tryptophan content of these proteins may be to small to be detected by tryptophan determination methods, including the one described here; c) coloration of lens nuclear material is not the result of destruction of tryptophan residues but may be caused by highly colored components which are not indoles and are not derived from protein-bound tryptophan.

Tryptophan might be involved in the process of nuclear coloration in a non-destructive sense, playing a role as UV-light sensitizer. Energy from the sun might be transmitted via tryptophan towards other compounds, present in the lens in bound and free state, causing them to decrease in concentration (radical scavengers, protein sulfhydryl groups) or to alter their structure (anthranilic acid, derived from a soluble free precursor; bityrosine, from protein-tyrosine).

In the next chapter (Chapter VII) we further discuss pos-

Table 6.II. TRYPTOPHANYL GROUPS FROM THE WATER- AND UREA-SOLUBLE FRACTION FROM NORMAL AND NUCLEAR-CATARACTOUS LENS PARTS.
The number of determinations are given in parentheses; data are expressed as mean ± SD.

Nuclear color	Age (years)	P-Trp (moles/moles protein‡)		
		Co	Nu	ENu
Normal	60 ± 18	2.6 ± 0.2 (6)	2.5 ± 0.3 (4)	
	65 ± 15			2.4 ± 0.2 (6)
Yellow	66 ± 14	2.7 ± 0.5 (12)	3.0 ± 0.5 (10)	
	71 ± 13			2.4 ± 0.5 (6)
Brown	71 ± 6	2.3 ± 0.3 (5)	2.0 ± 0.2 (4)	
	74 ± 7			2.1 ± 0.5 (6)
Dark brown	68 ± 10	2.5 ± 0.5 (8)	2.5 ± 0.7 (6)	
	75 ± 11			2.4 ± 0.6 (6)
Nigra	65 ± 15	2.8 ± 0.8 (5)	2.5 ± 1.0 (4)	
	79 ± 9			2.8 ± 0.6 (4)

Abbrevations used are: Co, cortex; Nu, nucleus; ENu, embryonic nucleus; P-Trp, protein tryptophan residues. Statistical evaluation is given in the text (level of significance, 0.05).

‡Calculated assuming an average subunit molecular weight of 20,000 D.

sible changes in lens tryptophan content, applying pyrolysis mass spectrometry to the oldest lens part, the embryonic nucleus.

PYROLYSIS MASS SPECTRA, SULFHYDRYL AND TRYPTOPHAN CONTENT OF THE EMBRYONIC NUCLEI FROM ADULT HUMAN NORMAL AND NUCLEAR-CATARACTOUS LENSES

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PYROLYSIS MASS SPECTRA, SULFHYDRYL AND TRYPTOPHAN CONTENT OF THE EMBRYONIC NUCLEI FROM ADULT HUMAN NORMAL AND NUCLEAR-CATARACTOUS LENSES

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Key words Lens nucleus, Cataract, Pyrolysis mass spectrum, Sulfhydryl content, Tryptophan

Summary

Cataractous state-related alterations were studied by Curie-point pyrolysis low voltage mass spectrometry performed on the embryonic nuclei from adult normal and nuclear-cataractous human lenses. It was shown that the relative intensities of ion signals assigned to sulfur dioxide and, tentatively, to other sulfur oxidation products correlate with the increase in lens nuclear color. Since these ion signals may represent pyrolysis fragments from methionine sulfoxide, methionine sulfone, cysteic acid and disulfide compounds present in the parent material, it is concluded that progressive oxidation of sulfur compounds appears to take place during nuclear cataractogenesis. Automated pyrolysis mass spectrometry coupled with multivariate analysis of the spectral data by computer turned out to be a rapid method of characterizing submilligram samples of lens material.

The results were supported by data obtained by conventional determination of sulfhydryl and tryptophan groups.

Introduction

Nuclear-cataractous human eye lenses are divided into groups of increasing nuclear color based on in vivo and in vitro examination of the lens. Previous biochemical research in our laboratory [1,2] indicates a relationship between

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lens nuclear color and the increase in water-insoluble and water-soluble protein fractions. In particular, the nuclear part of this type of cataractous lens contains proteins that become progressively polymerized, oxidized, fluorescent and colored; these proteins also contain increasing amounts of deamidated, age-dependently degraded polypeptide chains and racemized amino acid residues (for a review, cf. Ref. 3) [4—16]. The most accepted theory about the development of nuclear cataract is based on the mere fact that part of the ultraviolet light emitted by the sun (more than 295 nm) can enter the lens through the cornea [17,18] and cause damage to lens components. The role of sulfhydryl groups in the maintenance of lens transparency has been widely studied (for a review, cf. Ref. 3). Only in a few cases has the importance of separating cataractous and non-cataractous lens parts been emphasized [8,19]. In our laboratory, and by others, the non-cataractous cortex is separated from the colored nucleus by peeling decapsulated lenses until the hard nucleus is obtained. In a previous communication we reported on changes in sulfhydryl content of the human lens with emphasis on cataractous state and cataract localization [20]. To confirm these findings we have been searching for techniques enabling us to investigate parent lens material. Raman spectroscopic measurements on intact lenses [21] seem to be promising in finding lenticular alterations. Another instrumental technique for fast characterization of native lens material turned out to be pyrolysis-mass spectrometry (pyrolysis-MS). On pyrolysis the highly complex (non-volatile) organic matter is thermally fragmented under high vacuum conditions, yielding a mixture of small fragment molecules. This mixture of largely volatile components, which is characteristic of the original material, is directly analyzed by mass spectrometry and results in a kind of fingerprint spectrum. This method, which requires only μg amounts of non-derivatized substances, has already been applied to the analysis of complex organic materials in many fields of biology, biochemistry and biomedicine [22,23].

Much effort has been invested in the development of rapid and reproducible analysis systems [24,25]. As spectra of structurally related substances, like the lens materials described here, show complex spectra which differ only in the relative intensities of mass peaks, accurate quantitative comparison is necessary. For this purpose, special computer programs have been developed by which large series of spectra can be handled [26].

Using a Curie-point pyrolysis-MS system, which is fully automated with respect to sample introduction, pyrolysis-MS analysis and data handling, we applied this method on the embryonic nuclei from adult normal and nuclear-cataractous human lenses. This nucleus, appearing as a morphological entity and representing a nearly constant weight fraction of the adult lens, seemed to be suitable for pyrolysis-MS investigation of cataractous state-related alterations.

Materials and Methods

Lenses. Human nuclear-cataractous lenses were obtained from operations at the Eye Clinic of the Free University of Berlin (F.R.G.). The lenses were classified in vivo by means of a slit-lamp into four groups of increasing nuclear color: lenses with a yellow nucleus, a brown nucleus, a dark brown nucleus and a

black nucleus, ophthalmologically termed cataracta nigra. Immediately after surgery and re-examination, lenses were stored in capped vials at −70°C. Transport to Nijmegen took place in a container filled with liquid nitrogen. To prevent lyophilization during storage (−80°C), lenses in capped vials were sealed in plastic tubings.

Normal, completely transparent lenses were obtained within 5 h of death from individuals of various ages from the Eye Hospital at Rotterdam, The Netherlands.

Preparation of lens parts. Lens parts were prepared depending on the localization of nuclear color. Lenses were slowly thawed on an ice-cooled glass plate covered with parafilm. After incision of the capsula at the anterior side, the transparent cortices were removed with a spatula, immobilizing the hard nuclei by means of a needle. This procedure took no more than 3 min per lens. Normal lenses were treated the same way, considering the inner 40—50% of the wet weight of the lens to be the nucleus. The isolated nuclei were trephinized (inner diameter, 4.5 mm) in order to obtain the embryonic nucleus. This part of the lens, which fairly constantly represents 14% (30 mg) of the wet weight of the lens, cracked out by itself when the method described was used. A quarter of the embryonic nucleus was taken to prepare samples for pyrolysis mass spectrometry analysis.

Chemical analysis. Sulfhydryl data from the water- and urea-soluble protein fraction from lens parts were gathered by methods described previously [20], which are slight modifications of methods described by Truscott and Augusteyn [6]. Tryptophan data from the same protein fraction were gathered according to methods described by Neitchev and Boudevska [28], using bovine serum albumin and tryptophan as standards. Protein was determined according to the method of Lowry et al. [29], after incubating samples overnight with 0.1 N NaOH, 2% (w/v) Na_2CO_3 at room temperature. Protein contents were calculated by reference to a standard curve constructed using bovine serum albumin or calf α-crystalline (0—200 μg).

Pyrolysis mass spectrometry. Embryonic nuclei from adult normal and nuclear-cataractous human lenses were dissected and one quarter (7.5 mg) suspended in 1 ml pH 7.2 buffer (0.01 M phosphate, 0.145 M Cl^-, 0.17 M Na^+) by mild ultrasonic treatment. Suspension of nigra nuclei was aided by one homogenization step using a small pestle. From the suspensions 5 μl samples were applied to ferromagnetic wires and the solvent evaporated under rotation in vacuo. The coated wires were then mounted in glass reaction tubes.

The automated pyrolysis mass spectrometry system used (Fig. 1) has been described in detail elsewhere [24,30]. In short, it consists of a sample changing device, a Curie-point pyrolysis reactor with high frequency generator (Fischer Labortechn. GmbH, 1.5 kW, 1.1 MHz), a quadrupole mass filter (Riber, QM17) with ion-counting detector and on-line minicomputer (D-116, Digital Computer Controls). Pyrolysis was accomplished by inductive heating of the ferromagnetic sample wire (Fe/Ni alloy) up to the Curie temperature (510°C) within 0.1 s. The total heating time was 0.8 s. The above described pyrolysis technique and conditions enable highly reproducible analysis of the samples [25]. The volatile pyrolysis products enter the ionization chamber via a heated expansion volume (150°C) and are ionized by low voltage electron impact

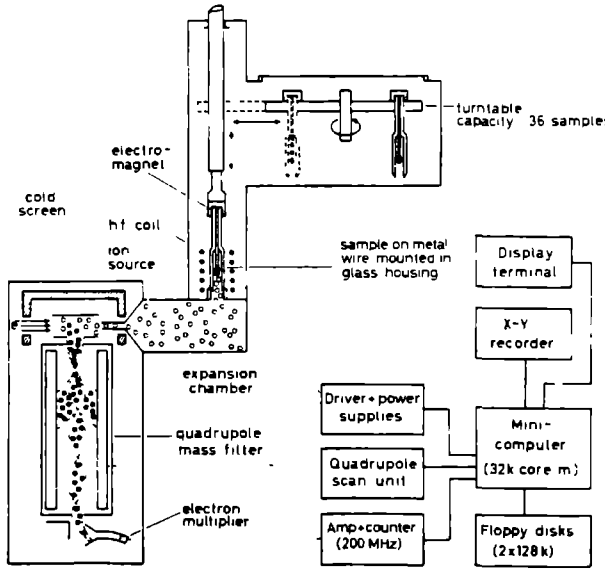


Fig. 1. Schematic diagram of the fully automated Curie-point pyrolysis mass spectrometry system.

(14 eV electrons). The mass spectrum of the resulting mixture of ions is repetitively scanned over the range 15—160 a.m.u. at a rate of 10 spectra/s during 20 s. All mass scans are accumulated and summed in the memory of the minicomputer by a signal averaging procedure, after which the averaged spectrum is stored on a floppy disk. Each embryonic nucleus sample was analyzed in quadruplicate. The following compounds were used as model substances: L-cystine, L-cysteine (Sigma); cysteic acid, methionine sulfoxide, methionine sulfone, methionine, L-Met-L-Met (Fluka); glutathione (Nutritional Biochemical Corporation) and Phe-Asp-Met-Trp-Gly-Met-Tyr (synthetic). Bovine serum albumin (Merck) and bovine α-crystallin were used as references.

Data processing. Processing of spectral data was carried out on a local CDC Cyber 7600 system. The first step in data handling consisted of a normalization procedure to correct for variations in sample size. Numerical comparison of the spectra was then carried out by multivariate analysis techniques described by Eshuis et al. [26]. For each embryonic nucleus an average spectrum and the standard deviation for each peak are calculated from the quadruplicate analyses. Every peak of the mass spectra was weighted by the ratio between inter-sample and mean intra-sample deviation. This ratio, the characteristicity value [26], is comparable to a Fisher ratio and, to some extent, to a signal-to-noise ratio. After this procedure the 20 masses with highest inter- to intra-sample deviation ratios were used to calculate a distance matrix, in which dissimilarities between the spectral patterns are represented by numerical values. These values were calculated using the modified Euclidean distance formula [26]:

$$d(X, Y) = \sqrt{\frac{1}{\sum w_i} \sum_{i=1}^{20} w_i \left(\frac{x_i - y_i}{\sigma_i}\right)^2}$$

wherein X ($x_1, x_2, ..., x_{20}$) and Y ($y_1, y_2, ..., y_{20}$) are two spectra, x_i and y_i are the intensity values of the 20 peaks with highest inter/intra sample deviation ratio, w_i (a weight factor for peak i), σ_i is the averaged standard deviation of peak intensity i, and d is the distance between both spectra.

The distance matrix provides the most complete information about the numerical relationships between the various spectra; however, because of the large number of distance values involved, it is not always easy to form a mental picture of the overall relationships. Therefore, a visual representation of all distance matrix data (data points in 20-dimensional feature space) is made by nonlinear mapping. This is an iterative computer procedure leading to a two-dimensional map which matches the original 20-dimensional configuration as closely as possible with respect to the relative nearest neighborship of all points. The imperfectness of fit with the real distance values is expressed as stress value.

Results

The embryonic nuclei involved in this study (Fig. 2) contained decreasing amounts of non-protein-bound sulfhydryl groups (chiefly glutathione as judged by amino acid analysis) in relation to the deepening of nuclear color (F, 17.0; degrees of freedom, 4,24; $P = 0.005$); the data are given in Table I. Cortex values were not significantly different (F, 1.61; degrees of freedom, 4,24; $P > 0.05$). Since wide variations were observed in the absolute values of the non-protein sulfhydryl groups in each lens part, we expressed the data in percentages of total non-protein sulfhydryl groups found in the embryonic nucleus and transparent cortex of the individual lens. The embryonic nucleus is constant in weight (30 ± 2 mg, 10 determinations) and represents 14% of the total lens wet weight. The weight percentage of the transparent cortex changes from 70 to 50% during progress of nuclear cataract as we noted earlier [20].

The free protein sulfhydryl groups of the water- and urea-soluble protein fraction of the embryonic nuclei and cortices showed less variation than the non-protein sulfhydryl groups (Table I). Since neither non-protein sulfhydryl nor protein sulfhydryl groups revealed age-related changes in the range of lens ages studied, mean data were tabulated. Comparison of the values for protein-sulfhydryl groups from transparent cortices and embryonic nuclei in relation to the cataractous state of the lens reveals that no changes occur to the protein sulfhydryl content of the transparent cortices from both normal and nuclear-cataractous lenses (F, 1.26; degrees of freedom, 4,37; $P = 0.30$). These results are in accordance with previous findings [20]. Decreases in protein sulfhydryl content occurred in the embryonic nuclei, related with the cataractous state; the protein sulfhydryl content of brown, dark brown and nigra embryonic nuclei was significantly different from that of normal nuclei (F, 3.48; degrees of freedom, 4,16; $P = 0.04$).

No significant changes (F, 1.42; degrees of freedom 4,23; $P > 0.05$) were found in the tryptophan content of the water- and urea-soluble proteins of the

TABLE I

SULHYDRYL AND TRYPTOPHAN DATA OF THE WATER- AND UREA-SOLUBLE FRACTION FROM CORTICES AND EMBRYONIC NUCLEI OF NORMAL AND NUCLEAR-CATARACTOUS LENSES

The number of observations are given in parentheses; data are expressed as mean ± S.D. Statistical evaluation is given in the text (level of significance, 0.05).

Nuclear color	Age (years)	Non-protein sulfhydryl (% of total)		Protein sulhydryl (mol/mol protein *)		Tryptophan content (mol/mol protein *)
		Cortex	Embryonic nucleus	Cortex **	Embryonic nucleus	Embryonic nucleus
Normal	65.2 ± 15.0	74.0 ± 5.3 (6)	30.6 ± 4.7 (6)	1.86 ± 0.19 (10)	1.27 ± 0.05 (2)	2.4 ± 0.2 (6)
Yellow	71.7 ± 13.3	58.6 ± 8.8 (6)	15.7 ± 5.0 (6)	1.90 ± 0.39 (13)	1.25 ± 0.62 (6)	2.4 ± 0.5 (6)
Brown	74.0 ± 7.0	67.9 ± 7.5 (6)	15.0 ± 4.1 (6)	1.66 ± 0.45 (6)	0.72 ± 0.30 (6)	2.1 ± 0.5 (6)
Dark brown	74.8 ± 10.5	70.1 ± 12.6 (6)	10.8 ± 6.0 (6)	1.84 ± 0.24 (7)	0.53 ± 0.03 (2)	2.4 ± 0.6 (6)
Nigra	78.6 ± 8.5	69.8 ± 14.8 (5)	10.3 ± 6.0 (5)	1.53 ± 0.60 (6)	0.32 ± 0.13 (5)	2.8 ± 0.6 (4)

* Calculated assuming an average subunit molecular weight of 20 000.

** Adapted from Ref. 20.

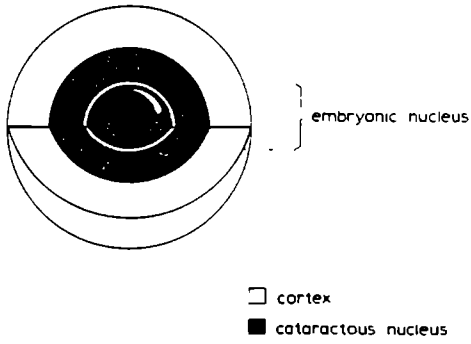


Fig. 2. Schematic representation of a human nuclear-cataractous lens.

embryonic nuclei from normal and nuclear-cataractous lenses; the results (Table I) are in accordance with earlier reports [19,31].

Embryonic nucleus preparations were further analyzed by pyrolysis-MS. Typical examples of spectra from embryonic nucleus samples from normal and nuclear-cataractous lenses are given in Fig. 3. The overall spectra of cataractous and normal embryonic nuclei show similar 'fingerprints'. However, significant cataractous state-related alterations in the relative intensities of some ion signals occur. Higher abundances of the ions at *m/z* 63, 64, 66 and 80 are found for cataractous lenses. These ions point to an increase of oxidized sulfur compounds (-SO-, $-SO_2-$, $-SO_3-$); they are also found in the pyrolysis mass spectra of methionine sulfoxide, methionine sulfone and cysteic acid (not shown). The main cataract-related intensity was found for *m/z* 64. Using pyrolysis high-resolution mass spectrometry it could be proven that the extra contribution to this mass value for the cataractous materials is due to the SO_2 ion. Also, the relative intensity of *m/z* 76, although very low, is positively correlated with the cataractous state. This fragment is tentatively assigned to CS_2 and may be derived from disulfide groups. The relative intensities of ions typical for proteins [22,27], viz. *m/z* 34 (H_2S derived from methionine, cysteine, cystine, etc.), *m/z* 48 (methanethiol, derived from methionine), *m/z* 117 and 131 (indoles, derived from tryptophan) and other aromatic fragments at *m/z* 94, 108, 120, 122 (phenolic compounds derived from tyrosine) and at *m/z* 92, 104, 117 (toluene, styrene and phenylacetonitrile derived from phenylalanine) show no obvious relationship with lens nuclear color. The relative intensities of the two characteristic ions, *m/z* 64 (SO_2) and *m/z* 80 (tentatively assigned to CH_3SO_2H or SO_3) are plotted in a scatter diagram for each cataractous state (Fig. 4). The differences in the relative occurrence indicate that during nuclear cataract development (i.e. nuclear color deepening) accumulation of oxidized sulfur-containing amino acid residues, such as methionine sulfoxide, methionine sulfone or cysteic acid, takes place. These compounds are difficult to study by use of biochemical techniques as is indicated by the work of Truscott and Augusteyn [4], but they can be detected readily by pyrolysis-MS. The rela-

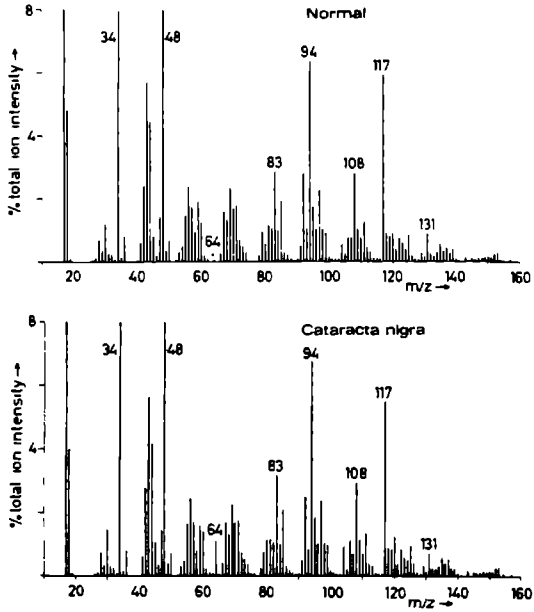


Fig. 3. Typical pyrolysis mass spectra of embryonic nuclei from normal and 'cataracta nigra' adult human lenses (averaged spectra from four determinations).

tionship between CS_2 (m/z 76) and the cataractous state is much less evident, but it may be argued that the amounts of non-protein-bound oxidized glutathione in the nucleus, which overwhelm the amount of protein disulfide bonds

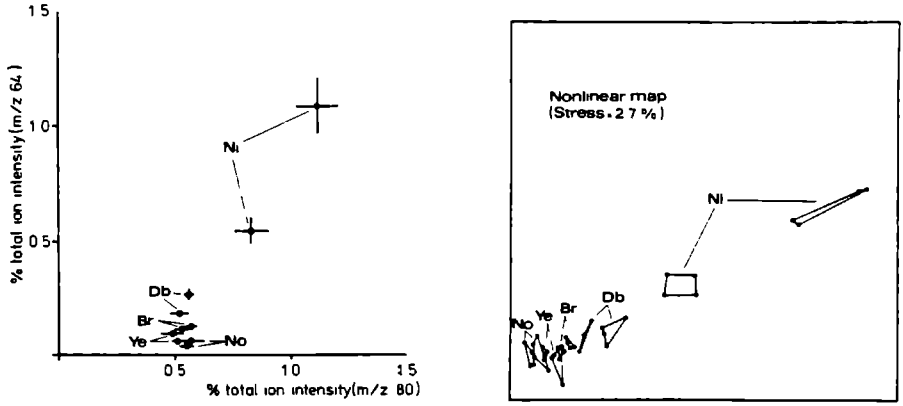
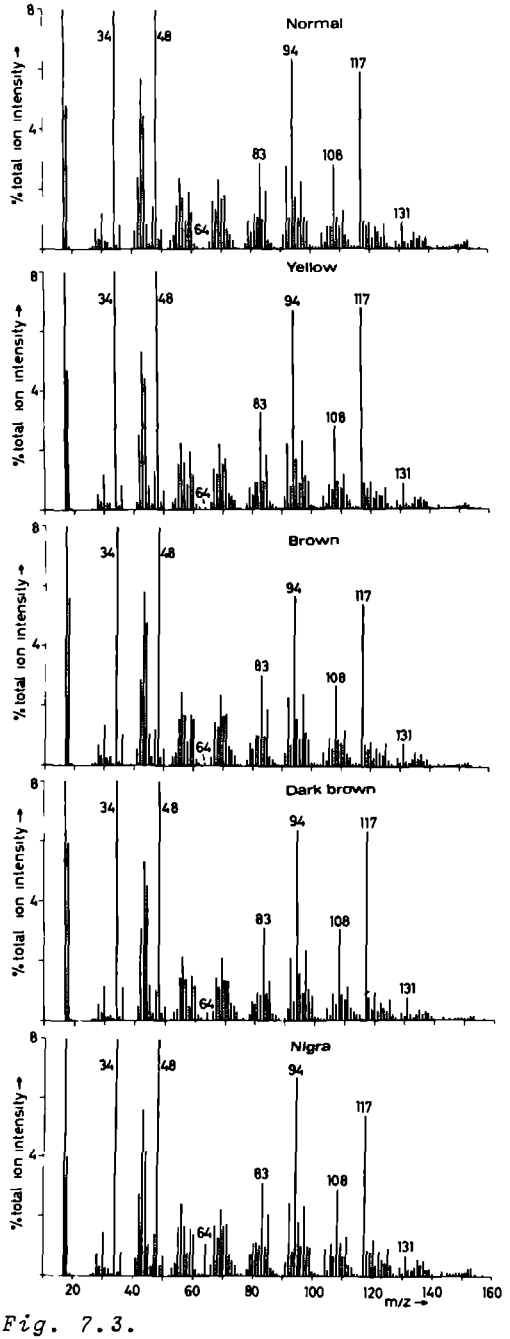


Fig. 4. Scatter diagram of ion intensities at m/z 64 and 80 in the spectra (average of four determinations) of the embryonic nuclei. Bars indicate standard deviations. No, normal; Ye, yellow; Br, brown; Db, dark brown; Ni, nigra.

Fig. 5. Non-linear mapping of relative distances (= differences) between the spectra of the embryonic nuclei. Each point represents a spectrum; the four duplicate spectra for each nucleus are connected. The low stress value indicates a good fit with the calculated distance relationships between the spectra. No, normal, Ye, yellow; Br, brown; Db, dark brown; Ni, nigra.



Chapter VII *Fig. 7.3.*

This figure has been added to the original publication.

TABLE II

SELECTION OF CHARACTERISTIC MASS VALUES FOR COMPARISON OF THE SERIES EMBRYONIC NUCLEI SPECTRA

Peak intensity parameters for 10 masses with greatest characteristicity are given.

m/z	% of total ion intensity	Mean intra-sample deviation	Inter-sample deviation	Characteristicity (w_1)
64	0.26	0.05	0.65	12.71
63	0.03	0.00	0.06	12.61
80	0.63	0.05	0.39	7.90
66	0.30	0.02	0.11	5.00
76	0.06	0.01	0.03	4.67
93	0.80	0.08	0.23	3.06
81	1.05	0.06	0.18	2.88
58	0.84	0.09	0.27	2.87
126	0.23	0.06	0.16	2.84
67	1.53	0.08	0.22	2.81

[6,20], show wide variations in individual lenses. As outlined above, relative intensities of single peaks can be used to discriminate between the various embryonic lens nuclei. To get a more comprehensive picture of the relationship between the embryonic nuclei spectra a numerical comparison was carried out by multivariate analysis, using 20 mass peaks with highest characteristicity (Table II). A distance matrix was calculated and, subsequently, the relative distance values were represented in the non-linear map given in Fig. 5. The map shows the data obtained from the complete series of embryonic nuclei and is mainly based on the mass peaks with highest characteristicity tabulated in Table II. It is evident from this map that a relationship exists between the spectra and lens classification, i.e. nuclear color. The similarity between the scattering of data points as observed in Figs. 4 and 5 indicates that the map mainly reflects the changes in relative peak intensities (*m/z* 64, 63, 80) which point to sulfur oxide components and that further differences between the spectra, e.g. due to interindividual variability, are of minor importance.

Discussion

Embryonic nuclei from normal and nuclear-cataractous adult lenses can be distinguished on the basis of their pyrolysis mass spectra. Accumulation of oxidized sulfur compounds (probably methionine sulfoxide, methionine sulfone or cysteic acid) and disulfide compounds seems to take place in relation to nuclear color. Decreases in free sulfhydryl groups (non-protein sulfhydryl and protein sulfhydryl) as found in the water- and urea-soluble embryonic nucleus fraction, seem to concur with the general idea that oxidation of sulfhydryl groups takes place during nuclear cataract development. In view of earlier findings [20], most alterations in proteins of the lens should be found in the urea-insoluble fraction which contains the colored proteins. No dramatic differences occur in protein structure and composition within each cataractous color group. The tryptophan content, as determined colorimetrically, seems not to be altered significantly upon nuclear cataract development. The findings

presented in this paper are in accordance with data obtained by chemical determination of sulfhydryl and tryptophan groups in the nuclear part of the lens [5,6,16,19,31].

The findings obtained by pyrolysis-MS are certainly not the result of artifacts produced by the analysis method Model experiments have shown that during pyrolysis no oxidation of sulfur compounds occurs by the action of the relatively large amount of ascorbic acid present in lens material In our opinion, this work demonstrates that pyrolysis mass spectrometry is a valuable tool in the analysis of human lenses, sample preparation is simple, sample analysis is fully automated and fast and the method requires only μg amounts of material. Highly reproducible spectra are obtained for individual human lenses. Differentiation between cataractous states might possibly be improved when, instead of color classification, spectral classification of lenses or embryonic nuclei becomes feasible.

It has been suggested [4,32] that oxidative damage to sulfur-containing residues in human lens protein and lens glutathione is simply the result of the action of oxygen or hydrogen peroxide on sulfur compounds.

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CHAPTER VIII

FATTY ACID ANALYSIS AND PEROXIDIZABILITY OF LIPIDS FROM NORMAL AND NUCLEAR-CATARACTOUS HUMAN LENSES

From: Experimental Eye Research, 1980, submitted

8. FATTY ACID ANALYSIS AND PEROXIDIZABILITY OF LIPIDS FROM NORMAL AND NUCLEAR-CATARACTOUS HUMAN LENSES

Summary

The possible role of lipid peroxidation in human lenses on aging and nuclear cataract development was studied. For the proportion of unsaturated fatty acids in the lipids from cortices or nuclei of lenses in the age range of 50 to 90 years no changes were detected. No cataractous state-dependent changes in the proportions of individual fatty acids were observed, either in the cortices or in the nuclei of nuclear-cataractous lenses. However, a significant difference was found between the proportion of unsaturated fatty acids in the cortex and the nucleus of each lens, except for cataracta nigra.

The lipid peroxidizability of the water-insoluble fractions was measured utilizing a carbon monoxide-hemoglobin binding test. In 25- to 90-year-old normal lenses no age-related alteration of peroxidation susceptibility was found. Nuclear-cataractous lenses revealed no significant differences in peroxidizability as compared with normal lenses.

Human nuclear-cataractous lenses constitute a small fraction of the total number of cataracts observed clinically in temperate areas, whereas dramatic increases in their percentage participation have been observed in areas where exposure to sunlight is high (Zigman, Datiles and Torczynski, 1979). Nuclear-cataractous human lenses can be divided into groups of increasing nuclear color, based on *in vivo* and *in vitro* examination of the intact lens (Kramps, Hoenders and Wollensak, 1976).

The most accepted theory about the development of nuclear cataract is dealing with the mere fact that part of the UV-light, emitted by the sun (>295 nm) can enter the lens through the cornea (Pitts, Cullen and Hacker, 1977). Aging of the lens and exposure to sunlight seem to determine when and where nuclear cataract is to develop. To date, damage to lens components is suggested to occur via free radical mechanisms involving tryptophan in a destructive sense (Zigman, 1977; Lerman and Borkman, 1979), as a sensitizer (Weiter and Subramanian, 1978) or as a generator of hydrogen peroxide (McCormick, Fischer, Pachlatko and Eisenstark, 1976) Evidence is missing to support the hypothesis that destruction of tryptophan is involved in the coloration of proteins from the human lens nucleus (Dilley and Pirie, 1974).

Production of lens fluorescence and pigmentation upon aging and nuclear cataract development (for a review, cf. Lerman and

Borkman, 1979) does not necessarily involve destruction of tryptophan due to photo-ionization (Amouyal, Bernas and Grand, 1979), as postulated by Borkman, Dalrymple and Lerman (1977). Light with wavelengths between 320 and 360 nm has been shown to produce a neutral tryptophan radical and a superoxide anion radical (Pailthorpe and Nicholls, 1971), but, according to Weiter and Subramanian (1978), tryptophan may also act as a sensitizer transferring energy from near-UV light to any receptor present in the lens. Accordingly, free radicals might be produced without destruction of tryptophan.

Sulfur compounds have achieved much attention in lens literature since these compounds can protect tissue against radiation and oxidation processes (Nagata and Yamaguchi, 1978; Slater, 1972). The sword of protection, however, is two-edged; in the lens the scavenging action of sulfur compounds may lead to disulfide bond formation in proteins, which process may lead, in addition to other events, to destabilization of protein structure (for a review, cf. Harding and Dilley, 1976; Garner and Spector, 1979). Glutathione and ascorbic acid*, both present in the lens in relatively high concentrations (for a review, cf. Kuck, 1975) , may protect lens components against oxidative challenge involving superoxide anions, hydroxyl radicals, hydrogen peroxide, singlet oxygen, lipid peroxides and lipid hydroperoxides (for a review, cf. Fridovich, 1978; Tappel, 1973; Feeney and Berman, 1976).

The question remains why only the oldest part of the lens changes color and reveals strong degeneration of fiber plasma

*non S-containing compound

membranes (Kobayashi and Suzuki, 1975), leaving the surrounding hull relatively unchanged. Recently, we could prove (van Haard, de Man, Hoenders and Wollensak, 1980a), in accordance with findings by others (Truscott and Augusteyn, 1977b), that the nucleus of even normal human lenses contains significantly lower glutathione levels than the corresponding cortex. We determined sulfhydryl groups in lens parts, separated on the basis of cataract localization, it was concluded that nuclear cataract develops from the inner nucleus towards the outer cortex, concomitantly with a decrease in the level of glutathione and protein sulfhydryl groups. In another report, we presented evidence that hydrogen peroxide could be involved in the production of lens fluorophors (van Haard, Hoenders and Ketelaars, 1980b). Action of hydrogen peroxide and radicals might explain earlier findings with respect to protein coloration and fluorescence enhancement in the lens, induced by irradiation and/or catalase inhibition (for a review, cf. Lerman and Borkman, 1979; Zigman, 1977; Kurzel, Wolbarsht and Yamanashi, 1973). The role of hydrogen peroxide in the production of methionine sulfoxide, occurring in nuclear-cataractous lens proteins (Truscott and Augusteyn, 1977a), has been suggested (van Haard et al., 1980b; van Haard, Hoenders, Wollensak and Haverkamp, 1980c).

Recently, it was suggested (Sternberg, Cotlier and Obara, 1978) that the increase of arachidonic acid ($C_{20:4}$) in lysophosphatidyl ethanolamine (LPE) found in senile cataractous human lenses, might play a role in cataractogenesis since this

fatty acid is peroxidation-susceptible. The formation of lipid-protein complexes in senile cataractous lenses as a result of peroxidation or other changes in fatty acids of the lens lipids may represent abnormalities in lens fiber membrane structure and composition; the lipid hydroperoxides formed may react with protein sulfhydryl groups and amino acid residues (Gardner, Kleiman, Weisleder and Inglett, 1977; Schaich and Karel, 1975). In fact, increasing amounts of oxidized and polymerized proteins are found in the urea-soluble (membrane-enriched) fractions in relation to the increasing nuclear color of the lens Kramps, 1977). The findings presented could substantiate the hypothesis that free radicals play an active role in lipid peroxidation damage to lens proteins in general, those containing sulfhydryl groups in particular. It seems worthwhile to investigate whether protein denaturation causes membrane degeneration in the nuclear region of the lens or whether these are two independent processes.

We investigated whether lipid peroxidation occurs during aging and nuclear cataractogenesis in the human lens. After a clear-cut separation of cataractous and non-cataractous lens parts, fatty acids were determined in each lens part. As control experiments whole lenses and water-insoluble (enriched plasma membrane) fractions were investigated. Moreover, we report on the lipid peroxidation susceptibility of the sonicated water-insoluble fractions from normal lenses and nuclear cataracts.

8.2. MATERIALS AND METHODS

8.2.1. Lenses

Lenses were obtained and handled as described in section 2.2.1.

8.2.2. Chemicals

All reagents were of analytical grade. Solvents were degassed by sonication and purged with nitrogen before use. Water was deionized and filtered using a Millipore system in combination with a mixed-bed ion-exchanger. If not stated otherwise, all solutions were prepared freshly and used within one day.

8.2.3. Preparation of water-insoluble lens fractions

Lenses were decapsulated and homogenized at 4°C in a pH 7.4 buffer (20 mM Tris-HCl, 80 mM NaCl), using 2 ml per lens. The homogenates were centrifuged once at 4°C (9,900 g, 25 min) to obtain the water-insoluble lens proteins including the fiber plasma membranes.

8.2.4. Preparation of lens parts

Lens parts were separated on the basis of the localization of nuclear color. Lenses were slowly thawed on an ice-cooled glass plate covered with parafilm. After incision of the capsule at the anterior side, the transparent cortices were removed with a spatula immobilizing the hard nuclei by means of a needle. This procedure took no more than 3 min per lens. As

reported earlier (van Haard et al., 1980a), the colored part increases in wet weight in relation to the deepening of nuclear color. Normal lenses were treated the same way, considering the inner 40-50% (wet weight) of the lens to be the nucleus. Isolated lens parts were kept on ice under nitrogen atmosphere to avoid oxidation by air.

8.2.5. Extraction of lens lipids

Lipid extractions were performed in the dark in a nitrogen atmosphere, according to a modified Folch-routine (Folch, Lees and Sloan-Stanley, 1957). Lenses, lens parts and lens fractions were homogenized in 0.1 M KCl at 4°C (5 strokes). Methanol was added and mixed with the suspension by two strokes of the pestle. At last a mixture of chloroform-methanol was added to obtain a total solvent to tissue ratio of 20:1 (v/w) and a final solvent ratio water: methanol: chloroform of 1:1:1 (v/v/v). The glass stoppered tubes, containing the homogenates were flushed with nitrogen and firmly closed. Extraction of lipids was performed by continuous shaking at room temperature for half an hour. After the extraction, the homogenates were centrifuged (5,000 g, 20°C, 10 min) and the chloroform layers isolated carefully. This procedure was repeated twice. The combined chloroform layers were washed once with 0.2 ml 0.1 M KCl and, if necessary, filtered through a glass filter to remove residual protein. The chloroform layers were evaporated under a stream of dry nitrogen.

8.2.6. Preparation of lens lipid liposomes

Lens lipid liposomes were prepared according to the following method. The modified Folch-routine (Folch et al., 1957) was applied to extract the major lipids from the water-insoluble lens fractions (see above). No anti-oxidant was added for obvious reasons.

The dry lipids were immediately shaken,under nitrogen into a micellar suspension in 1.5 ml pH 7.4 buffer (20 mM Tris-HCl, 80 mM NaCl) at 4°C. This procedure took 30 minutes.

8.2.7. Preparation of hemoglobin solution

Hemoglobin was prepared as follows: freshly obtained donor blood was diluted ten times with 0.9% NaCl (w/v) and centrifugated (5,000 rpm, 10 min). Cells were washed three times with saline solution. The buffy coat was removed by aspiration. After lysis of cells by hypotonic shock (water: CCl_4, 1.5: 0.5, v/v) under continuous shaking, the cell debris was removed by repeated centrifugation (10,000 rpm, 10 min). Hemoglobin solutions were stored frozen and diluted before use. For optimal binding hemoglobin solutions were used within one week.

8.2.8. Lipid peroxidation experiments

The water-insoluble pellets were resuspended in 1.5 ml buffer by external sonication on ice at equal distance from the vessel wall. The suspensions were transferred to 3 ml vials, capped with Teflon valves (Pierce Chem. Co., Rockford,

Ill.). As a sample, 0.5 ml suspension was used and another equal volume as the reference. With the liposome dispersions the same procedure was followed. The remaining suspension was used to determine inorganic phosphate concentrations according to Bonting (1970). A conversion factor of 25 was used to relate the amounts of phosphate to phospholipids.

Lipid peroxidation was initiated by addition of a buffered ferric ammonium sulfate and ascorbic acid solution (pH 7.4) to the sample vial (Wolff and Bidlack, 1976). The final concentrations were 0.33 mM ferric sulfate and 1.66 mM ascorbic acid in 20 mM Tris-HCl, 80 mM NaCl, pH 7.4. To the reference vials only buffered EDTA (final concentration, 2 mM) was added. Lipid peroxidation was allowed to continue during 30 min. Samples and references were incubated at 37°C with occasional shaking. To stop the peroxidation reaction, EDTA was added to the samples; to the reference the initators were added.

A buffered human hemoglobin solution (pH 7.4) was gently mixed into both the sample and the reference solution to a final concentration of 5 µM. One minute was allowed to assure maximal carbon monoxide binding. The difference spectra were recorded at 37°C utilizing a Cary 118 spectrophotometer in the split beam mode. Using the optical density at 421 nm minus that of the isosbestic point at 480 nm, an extinction coefficient of 58.7 mM^{-1} cm^{-1} was determined for carboxy-hemoglobin which is in accordance with the value reported by Wolff and Bidlack (1976).

8.2.9. Fatty acid analysis

Fatty acids from the lipids were analyzed as their methylesters by means of gas liquid chromatography. Fatty acid derivatization was performed using 14% BF_3-methanol (w/v) according to Morrison and Smith (1964). Reliability of this method was tested using standard fatty acid mixtures (Supelco, Inc., Bellafonte, Pa). After quickly cooling, a small amount of anhydrous Na_2SO_4 was added and the methylesters extracted with pentane. The extract was concentrated under nitrogen. Internal standards, $C_{15:0}$- or $C_{23:0}$-methylesters, were added and all fatty acid methylesters redissolved in anhydrous pentane. A Pye Unicam series 204 chromatograph, equipped with a flame ionization detector was used at 210°C isothermally. Samples (1 µl) were analyzed on a 2.1 m x 4 mm i.d. coiled glass column packed with 10% SP-2330 on Supelcoport 100-120 mesh (Supelco, Inc., Bellafonte, Pa). Chromatographic conditions were: injector temperature, 250°C, detector temperature, 250°C, carrier gas (nitrogen) flow rate, 30 ml/min. Peak areas, retention times and fatty acid weight percentages were measured and calculated using a Hewlett-Packard 3380A integrating unit. Identification of the methylesters was performed by comparing retention times with those of standards, by plotting retention times (logarithmic) versus carbon number for identification of unknown fatty acids and, finally, by hydrogenation of samples using Wilkinson catalyst in benzene (tris-triphenylphosphine rhodium chloride) and rechromatography.

Analysis of fatty acid methylesters from lipids extracted

from the water-insoluble lens fractions was performed on 3% EGSS-X on Gas-Chrom Q (100-120 mesh) at 190^{o} isothermally.

Fractions eluting at 22.6 min and near 40 min on the SP-2330 column were not taken into account since they were not hydrogenated or revealed anomalous behavior after hydrogenation.

The amount of urea-insoluble material, including that of the fiber plasma membranes, is very low in normal human lenses. The amounts of urea-insoluble material increase in relation to the cataractous state, most of it representing denaturated proteins (Kramps et al., 1976; Kramps, 1977). In order to compare fatty acid analyses of the lipids in intact and disrupted plasma membranes from normal and nuclear-cataractous lenses and to obtain more data on the proteins entrapped in the fiber membranes, we studied the water-insoluble lens fractions which increase in weight percentage both during aging (for a review, cf. Garner and Spector, 1979) and nuclear cataract development (for a review, cf. Harding and Dilley, 1976).

The cortex of normal and nuclear-cataractous lenses (60 to 80 years of age) contains twice as much phospholipids as the nucleus; no cataractous state-dependent changes were found (Kramps, 1977). To further investigate these findings we performed fatty acid analyses on whole lenses, lens parts and water-insoluble fractions.

The lipids from water-insoluble human lens fractions contain a fairly constant weight percentage (about 50%) unsaturated fatty acids as is shown in Table I. No obvious relationship between the proportion of any fatty acid and the cataractous state was found. The percentages of polyunsaturated fatty acids were very low, in most cases less than 6% of the total weight.

Table I *FATTY ACID COMPOSITION OF THE LIPIDS FROM THE WATER-INSOLUBLE FRACTION OF NORMAL AND NUCLEAR-CATARACTOUS HUMAN LENSES*+*

		NUCLEAR COLOR			
Fatty acid	Normal	Yellow	Brown	Dark brown	Nigra
14:0	3.0	2.5	2.0	2.5	4.0
16:0	35.5	33.5	31.0	31.0	31.5
16:1	1.0	0.5	0.5	0.5	1.0
18:0	2.0	1.5	2.0	1.5	2.5
18:1	15.5	12.0	14.0	12.0	11.0
18:2	tr	tr	tr	tr	tr
20:0	0.5	0.5	0.5	0.5	0.5
20:1	0.5	0.5	0.5	0.5	0.5
20:3	0.5	0.5	0.5	0.5	0.5
20:4	0.5	0.5	0.5	0.5	1.0
22:0	3.5	3.5	3.5	3.0	5.0
22:1	3.0	3.5	2.5	2.5	4.0
22:4	0.5	1.0	1.0	1.5	1.0
23:1	1.0	0.5	0.5	1.5	1.0
24:0	5.5	7.0	7.5	6.5	6.0
24:1	26.0	30.5	29.0	31.5	28.5
22:6	1.0	2.0	4.0	3.0	2.5
% polyunsaturates	2.5	4.0	6.0	5.5	5.5
% unsaturates	49.6	51.5	53.0	53.0	51.5
Weight ratio 18:0/18:1	0.13	0.13	0.14	0.14	0.23

*Lenses ranged in age from 60 to 80 years, 10 lenses were used for each determination

+Values are expressed as weight percentages

The absolute amounts of fatty acids per lens (200 µg in the water-insoluble fraction of normal lens) revealed a strong decrease (up to 80%) with the onset of nuclear cataract. Since the data obtained were derived from determinations on pooled

lens material (10 lenses), no statistical evaluation is given. The ratios of $C_{18:0}/C_{18:1}$ in water-insoluble lipids were 0.13-0.14 except for cataracta nigra (0.23), whereas in the water-soluble fractions twice as high ratios were found.

When decapsulated individual lenses of nearly the same age and with increasing nuclear color were analyzed, we found a fairly constant weight proportion unsaturated fatty acids of 36% (Table II). A rather unexpected finding in these lenses

*Table II FATTY ACID COMPOSITION OF LIPIDS FROM INDIVIDUAL LENSES AND LENS PARTS DIFFERING IN CATARACTOUS STATE**

				Fatty acid data		
				% Unsaturates∞	Ratio	
Nuclear color	Lens region∝		Age (yrs)		18:0/18:1	16:0/18:1
Normal	whole	(1)	75	36	1.20	1.29
	Nu	(3)	>56.7±14.8	33.4±1.0	1.16±1.11	1.99±0.58
	Co	(3)		53.8±1.5	0.51±0.22	0.44±0.02
Yellow	whole	(1)	76	33	1.40	1.62
	Nu	(3)	>54.7± 5.5	31.9±8.9	2.02±1.29	2.05±1.00
	Co	(3)		51.8±0.6	0.45±0.08	0.67±0.11
Brown	whole	(1)	76	37	1.24	1.26
	Nu	(3)	>65.0±10.0	33.4±2.7	1.71±0.53	1.97±0.20
	Co	(3)		52.4±4.3	0.52±0.18	0.68±0.13
Dark brown	whole	(1)	76	35	1.33	1.64
	Nu	(3)	>77.7± 7.0	31.5±7.1	1.95±0.59	2.60±0.51
	Co	(3)		54.5±9.9	0.51±0.39	0.64±0.19
Nigra	whole	(1)	78	42	0.55	0.71
	Nu	(3)	>68.7± 9.3	35.9±6.3	2.21±1.16	2.79±1.42
	Co	(3)		46.5±7.1	0.74±0.34	0.84±0.13

*Values are expressed as mean ± S.D.

∝Abbreviations used are: Nu, nucleus; Co, cortex. The number of determinations is given in parentheses.

∞Values are expressed as weight percentages.

and also in their parts (Table II) was the unusually large proportion of $C_{18:0}$ fatty acid in comparison with levels found in the water-insoluble fractions (Table I). Since plasmalogens are known to be present in lens lipids (Broekhuyse, 1968; Kuck, 1975), we suspected the presence of dimethyl acetals, produced by the acid methanolysis. However, comparison of chromatographic patterns with those of Anderson, Benolken, Kelleher, Maude and Wiegand (1978), who utilized the same SP-2330 column, revealed that dimethyl acetals represented less than 1% of the total peak areas and could be discerned from the $C_{18:0}$ peaks.

Fatty acid analyses of the lipids of lens parts (Table II), derived from individual human lenses by clear-cut separation of cataractous and non-cataractous lens parts, revealed no age-related phenomena within the range of lens ages studied (49-85 years). Since no correlation between age and fatty acid data was obtained, mean values were calculated. Figure 1 illustrates a constant difference between the gas liquid chromatographic patterns obtained from cortical and nuclear fatty acid methyl-esters. Nuclear patterns revealed a high $C_{18:0}$ and a low $C_{18:1}$ peak, whereas in cortical patterns the situation was reversed. Neither the nuclear nor the cortical data revealed a significant relationship with the cataractous state (α=.05; one-way analysis of variance). However, differences existed between the percentage unsaturated fatty acids from cortices and nuclei. The cortices contained significantly higher percentages unsaturated fatty acids than the nuclei in all groups of lenses and in each lens ($P<0.02$; two-tailed paired t-test); this dif-

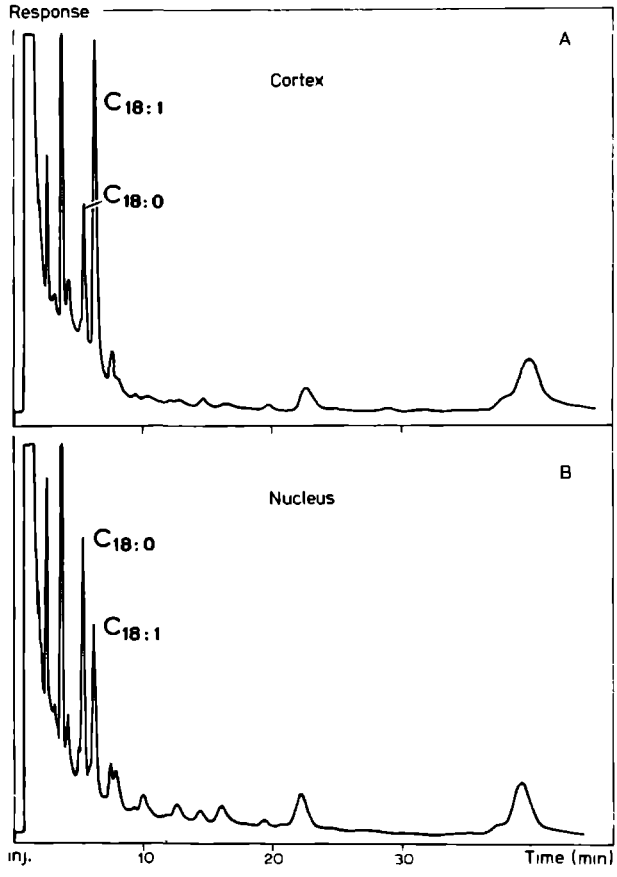

Fig. 8.1. Typical gas liquid chromatograms of fatty acids of cortical (A) and nuclear (B) lipids from individual human normal and nuclear-cataractous lenses.

ference became insignificant at the most severe state of cataract (nigra).

Liposomes prepared from normal human lens lipids turned out to be a bad source for the production of carbon monoxide. After 2 h incubation, less than 1 nmole CO/mg phospholipid was produced by liposomes derived from normal human lenses, ranging in

age from 27 to 84 years.

In order to compare normal with nuclear-cataractous lens membranes, we chose the water-insoluble fraction for further investigation since this fraction should contain intact membranes in addition to disrupted ones. As indicated in Figure 2, formation of CO within 30 minutes was rather constant when

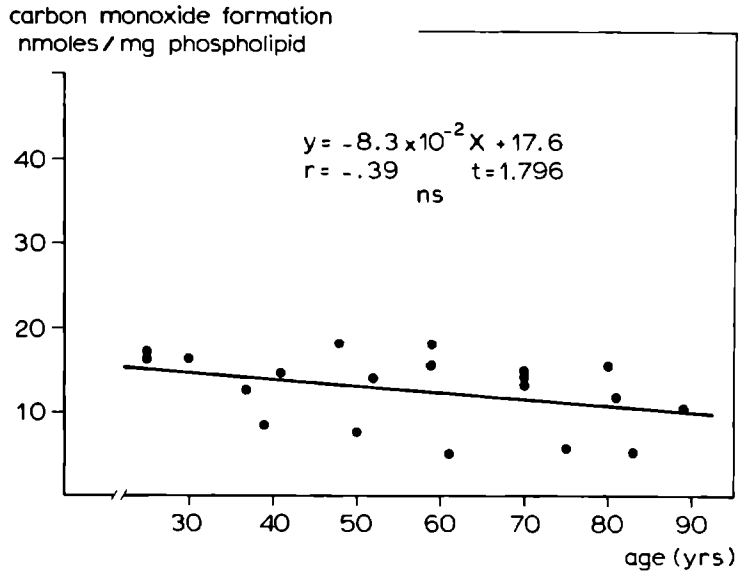

Fig. 8.2. Carbon monoxide formation as a function of age by the sonicated water-insoluble fraction from normal human lens.

investigating water-insoluble fractions from normal human lenses, ranging in age from 25 to 90 years. Up to 50 times more CO/mg phospholipid was produced by the sonicated water-insoluble fractions than by the corresponding liposomes. Comparing lipid peroxidation susceptibilities of the sonicated water-insoluble fractions from normal lenses and nuclear cataracts

(Table III), it was found that samples of advanced nuclear cataracts did not produce significantly different amounts of carbon monoxide than those of normal lenses of similar age. Only yellow samples produced significantly less carbon monoxide than normal samples (Student's t-test; $t_{0.05}$; two-tailed).

Table III *NON-ENZYMATIC CARBON MONOXIDE PRODUCTION BY SONICATED WATER-INSOLUBLE FRACTIONS FROM NORMAL AND NUCLEAR-CATARACTOUS HUMAN LENSES**

Nuclear color	Age (years)	Carbon monoxide production (nmoles/mg phospholipid)∞
Normal	72 ± 12	12.77 ± 4.07 (11)
Yellow	65 ± 7	4.13 ± 2.23 (3)
Brown	72 ± 9	8.43 ± 3.31 (4)
Dark brown	68 ± 13	10.43 ± 1.19 (3)
Nigra	80 ± 11	10.77 ± 1.53 (3)

*Values are expressed as mean S.D.

∞Carbon monoxide was generated within 30 min at 37°C by a mixture of ferric ammonium sulfate and ascorbic acid (see Materials and Methods). Statistical evaluation is given in the text (level of significance: 0.05). The number of determinations is given in parentheses.

The mammalian lens is equipped with several defense systems against oxidative challenge. It is striking that all enzymatic defense activities are located in the outer regions of the lens (Kuck, 1977; Varma, Ets and Richards, 1977; Bhuyan and Bhuyan, 1978; Crouch, Priest and Duke, 1978; Rogers and Augusteyn, 1978). The protein and membrane synthesis systems may be well protected against damage caused by various oxidants generative *in vivo*, like hydrogen peroxide, superoxide anions, lipid peroxides, lipid hydroperoxides, singlet oxygen and hydroxyl radicals. In the nuclear region of the human lens no protein synthesis occurs (Dilley and van Heyningen, 1976) and this part of the lens tissue seems to be only protected by glutathione and ascorbic acid. It is now well established that during nuclear cataract development lens protein sulfhydryl groups have been oxidized to form disulfide bonds (for a review, cf. Harding and Dilley, 1976), presumably caused by free radicals. It would be interesting to know whether unsaturated fatty acids in the plasma membrane lipids, especially in the nuclear region, are a target for free radicals or other oxidants generated *in vivo*.

The lipid composition of the normal human lens was determined by Feldman (1967). Lipid class compositions of old human normal and cataractous lenses at different age have been described (for a review, cf. Kuck, 1975). The fatty acid composition of some lipid classes is known (Sternberg, Cotlier and

Obara, 1978; Broekhuyse, 1972; 1974; Tao and Cotlier, 1976; Obara, Cotlier, Kim, Lueck and Tao, 1976a). Only eight fatty acids are quantitatively important in the total lens lipids, including $C_{16:0}$, $C_{16:1}$, $C_{18:0}$ and $C_{18:1}$ (Kuck, 1975). Phospholipids and sphingolipids each make up about a third of the total lipids. In the 70 year old human lens, sphingomyelins represent up to 65 and 75% of the total cortical and nuclear phospholipids, respectively (Broekhuyse, 1974), and form compact complexes with cholesterol (Obara, Cotlier, Lindberg and Horn, 1976b). Sphingomyelins also represent the major part of the sphingolipids occurring in the lens (Kuck, 1975).

During aging of the lens the amounts of sphigomyelins seem to increase, whereas the total amount of phospholipids decreases both in cortices and nuclei (Broekhuyse, 1974). Sphingomyelins are characterized by high proportions of $C_{16:0}$, $C_{24:1}$ (tentatively assigned) and $C_{18:1}$ (in lens epithelium) (Broekhuyse, 1974). The proportion of unsaturated fatty acids in sphingomyelins ranges from 45 to 65%, depending on the lens part studied. The levels of unsaturated fatty acids in another phospholipid, lysophosphatidyl ethanolamine (LPE), from old and senile cataractous human lenses are about 15 and 32%, respectively (Sternberg et al., 1978). These lipids make up about 11 and 18% of the total phospholipids of cortices and nuclei, respectively, from 70-year-old human lenses (Broekhuyse, 1974). Lysophosphatidyl ethanolamines, like other lysophospholipids, may form complexes with cholesterol, depriving the latter of its structural function in maintaining membrane integrity

(Reman, Demel, de Gier, van Deenen, Eibl and Westphal, 1969); they are characterized by high levels of $C_{16:0}$, $C_{18:0}$ and, in cataractous lenses, of $C_{20:4}$, $C_{24:0}$ and an unknown fatty acid (Sternberg et al., 1978).

Fatty acid analyses of water-insoluble fractions from normal and nuclear-cataractous lenses (this study) revealed high values of $C_{16:0}$, $C_{18:1}$ and $C_{24:1}$, these data may reflect the findings of Sternberg et al. (1978) in that up to 45% of the cortical and up to 34% of the nuclear lysophosphatidyl ethanolamines are water-soluble in their set-up, leaving other phospholipids (mainly sphingomyelins) in the water-insoluble fraction. Since the percentage of LPE varies between 10 and 20% of the total phospholipids in cortices and nuclei of the old human lens, their fatty acids do not attribute much to the findings for the water-insoluble lens fractions. Therefore, we cannot confirm the results of Sternberg et al. (1978) stating that in senile cataractous lenses accumulation of arachidonic acid ($C_{20:4}$) takes place, rendering the lens peroxidation-susceptible. In water-insoluble fractions from nuclear-cataractous lenses we did not find increased levels of arachidonic acid ($C_{20:4}$) and no obvious decreases in $C_{16:0}$ or $C_{18:0}$ (Table I). In individual normal and nuclear-cataractous lenses the same data were obtained, except that in nigra lenses a decrease in $C_{16:0}$ and $C_{18:0}$ was found (Table II). Conflicting data, however, may be well explained by the low percentages of LPE in comparison with sphingomyelins in old human lenses; it should, therefore, be argued that one has to investigate individual lipid

classes from individual human lenses and lens parts.

From earlier experiments in our laboratory (van Haard et al., 1980a) it has become evident that during nuclear cataractogenesis the colored and hard inner part of the lens increases in weight, roughly from 30 to 50%, depending on the cataractous state. It has been reported (Ehlers, Matheissen and Andersen, 1968) that 50% of the lens wet weight at old age represents the newborn lens wet weight; by several others (Pirie, 1968; Truscott and Augusteyn, 1977b) this part of the lens at old age has been regarded as the nucleus. From our findings it was concluded that nuclear cataract grows inside-outside until more than 50% of the lens wet weight has changed color and consistence at the most severe state of cataract.

In order to study the aging of the plasma membranes, we separated cortices and pigmented nuclei on the basis of cataract localization and analyzed the fatty acids of lipids from these lens parts. Since the nuclei isolated gain volume upon nuclear cataract development (van Haard et al., 1980a), the fatty acid data from the nuclei at the most severe stage of cataract may approach cortical data. At the preceding stages of nuclear cataract we found significant differences in the proportions of unsaturated fatty acids between cortices and nuclei (Table II). Within the same range of lens age, we found no evidence for a change in the proportion of unsaturated fatty acids upon development of nuclear cataract, either in the cortical or in the nuclear region of the lens. Since cortical membranes may contain high amounts of $C_{18:1}$ (Broekhuyse, 1974),

our data may simply reflect aging and metabolism of plasma membranes within each lens. These processes may result in decreased fluidity of lens plasma membranes due to changes in their levels of $C_{16:0}$ and $C_{18:0}$ at the cost of $C_{18:1}$, leading to the high cholesterol/phospholipid ratios as found in old calf lens fibers (Broekhuyse, Kuhlmann, Bijvelt, Verkley and Ververgaert, 1978; Broekhuyse, Kuhlmann and Yap, 1979). Moreover, data from early stages of nuclear cataract may reveal differences in the unsaturated fatty acid content of cortices and nuclei as a consequence of the way we isolated the colored lens parts. However, the absolute amounts of fatty acids from the plasma membranes (this study, water-insoluble fraction) seem to decrease during nuclear cataract development, but not the total lens lipid content (unpublished results). The absolute amounts of phospholipids in the nuclear region are lower than in the cortical region (Kramps, 1977), but no obvious change in any fatty acid proportion occurs during nuclear cataract development. We can, therefore, not conclude that fiber membrane structure alterations, as seen in nuclear-cataractous lenses, are accompanied by dramatic proportional changes in their fatty acid composition due to lipid peroxidation. On the contrary, intact membranes seem to decrease in amount but remain age-dependently constant in composition. Alterations in individual lipid classes, however, may have strong effects on lens membrane structure without being detected in an overall study such as this one.

Kurzel et al. (1973) attempted to detect lipid peroxidation products in human lenses using the thiobarbituric acid test

(Pryor and Stanley, 1975). This test is mainly designed for the detection of malondialdehyde which is a major peroxidation product from polyunsaturated fatty acids. However, as we have shown, human lens plasma membranes contain very little polyunsaturated fatty acids. Experimental evidence from our laboratory adds belief to reports (Gutteridge, 1977; Uchiyama and Mihara, 1978) that several problems can arise using the thiobarbituric acid test for measuring lipid peroxidation, in particular in protein-rich samples like lens tissue.

In view of the absence of heme groups in the lens, which are thus unable to interfere, we utilized a specific test involving non-enzymatically generated carbon monoxide from water-insoluble lens fractions. In order to measure the lipid-peroxidation susceptibility of human normal and nuclear-cataractous plasma membranes, the carbon monoxide generated was bound by hemoglobin (Wolff and Bidlack, 1976). This test does not need polyunsaturated fatty acids per se (Wolff and Bidlack, 1976). Results of our study on pseudo membrane structures add to the suggestion that no lipid peroxidation has occurred in human lens plasma membranes; there is no prevalence for severe cataracts to produce more lipid peroxidation product (CO) than normal lenses.

Lens plasma membrane degeneration in the nuclear region of the lens, as seen by electron microscopy (Kobayashi and Suzuki, 1975), seems not to be caused by radical processes within the membranes, but radicals might be responsible for alterations occurring to the lens proteins (for a review, cf. Lerman and Borkman, 1979; Harding and Dilley, 1976). The integrity of lens

plasma membranes seems to be damaged by age-dependent processes like accumulation of cholesterol, lysophospholipids and sphingomyelins within the membranes, followed by segregation; these changes may lead to increased imbibement of aged lens proteins (Obara et al., 1976a, b; Broekhuyse et al., 1978; Sternberg et al., 1978). During nuclear cataract development the oxidized and polymerized proteins may further disturb the membrane structure, rendering its disintegration secondary to the cataractous process (Broekhuyse, 1974). Structural changes in human lens nuclear plasma membranes during aging may cause decreased permeability (and thus decreased concentration) of radical scavengers like glutathione and ascorbic acid, which are necessary to protect aging proteins against damage (Truscott and Augusteyn, 1977b; Anderson and Spector, 1978; van Haard et al., 1980a). Aging of lens proteins and lens plasma membranes seems to determine when and where nuclear cataract is to develop under the influence of the sun as indicated by the findings of Zigman et al. (1979). Nuclear cataract develops inside-outside (van Haard et al., 1980a) since the inner part of the lens is challenged continuously by UV-light under conditions where radicals are likely to be generated, namely at age-dependently lowered radical scavenger concentrations.

VERY ACIDIC POLYPEPTIDE CHAINS FROM OLD HUMAN EYE LENSES 216

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Very Acidic Polypeptide Chains from Old Human Eye Lenses

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Key Words. Human lens · Aging Nuclear cataract · Very acidic polypeptide chains · Degradation · Deamidation

Abstract. The very acidic polypeptide chains found in aging human eye lenses are products of post-synthetic alterations known to occur in mammalian eye lens proteins. Isoelectric focusing in the presence of urea as well as dodecyl-sulphate gel electrophoresis reveals very diffuse bands indicating narrow range heterogeneity in charge and molecular weight. The very acidic chains are derived from α-crystallin subunits as indicated by their primary structure and electrophoretic behaviour. During nuclear cataract development the very acidic polypeptides play a role in the protein aggregation process.

Introduction

During the past year we have investigated the polypeptide composition of the water-soluble crystallins from old human eye lenses, to determine whether they are subject to similar post-synthetic alterations known to occur in bovine lens crystallins [5, 6]. Mainly α-crystallin has been studied from a quantitative point of view (for a review, see *Harding and Dilley* [3]). Post-synthetic deamidation and degradation processes of α-crystallin subunits occur next to an age-dependent increase in molecular weight of α-crystallin aggregates. The relationship between shortening of polypeptide chains and increase of molecular weight has been postulated [17]; the elucidation of this problem is clearly hampered by the enormous charge and size heterogeneity observed even in relatively young material like calf lens cortical α-crystallin [16].

It is tempting to speculate that degradation processes in both A- and

B-type α-crystallin chains may lead to the loss of contact regions between the subunits [15], possibly leading to polymerization towards higher molecular weight and even insoluble aggregates

In the water-insoluble and, more pronounced, in the urea-insoluble lens fraction from old human lenses huge aggregates are found containing polypeptide chains that have undergone various age-related processes, partly quite similar to those found in bovine lens [13, 14] The nuclear region of older human lenses contains decreased amounts of low molecular weight α-crystallin as compared to the cortex, suggesting that α-crystallin has become insoluble [5, 12] We have investigated the very acidic polypeptide chains found in old human lenses to determine whether they are products of post-synthetic alterations

Materials and Methods

Nuclear cataractous lenses, ranging in age from 50 to 90 years, were obtained from operations at the Eye Clinic of the Free University, Berlin (FRG) The lenses were classified *in vivo* according to colour by slitlamp microscopy Normal lenses were obtained within 5 h after death of individuals from the Eye Hospital at Rotterdam, The Netherlands

Lens extracts were prepared as described in an earlier publication [1] Water-soluble low-M_r α-crystallin and high-M_r crystallins were isolated by means of gel chromatography on Sephacryl S-200 and Bio-Gel A-5M Lyophilization was avoided until the final isolation step

The very acidic chains were isolated from 100 human cataractous lenses (yellow nucleus) after dialyzing water-soluble and insoluble lens fractions against 7 *M* urea, 0 02 *M* NaOAc, pH 5 0 at 4 C After centrifugation (9,900 *g* 25 min) to remove urea-insoluble material, supernatants were applied to a carboxymethylcellulose column (CM-52, Whatman) A linear gradient (2 × 200 ml) from 0 02 to 0 150 *M* NaOAc, pH 5 0 containing 7 *M* urea was applied The protein fraction eluting at 0 05 *M* NaOAc, pH 5 0 was collected, desalted and lyophilized Further efforts to fractionate the very acidic chains by means of ion-exchange chromatography were unsuccessful

Tryptic and chymotryptic hydrolysis, peptide mapping and amino acid analyses were performed according to *de Jong and Terwindt* [4] C-terminal amino acid sequence determination was performed according to *Laine et al* [8]

Isoelectric focusing (pH 3 5 10, 5–8 and 2 4–4) in the presence of urea was performed as described before [6] Stain percentages were calculated by cutting and weighing Since the primary structure of some occurring peptides is not known, no corrections were made for binding of the staining agent SDS gel electrophoresis was performed according to *Laemmli* [7] using 15% gels Sedimentation analysis was performed in a Beckman Spinco E analytical ultracentrifuge using Schlieren optics at 60,000 rpm Antisera against human eye lens proteins were raised in New Zealand White rabbits Double diffusion tests were performed as described before [10]

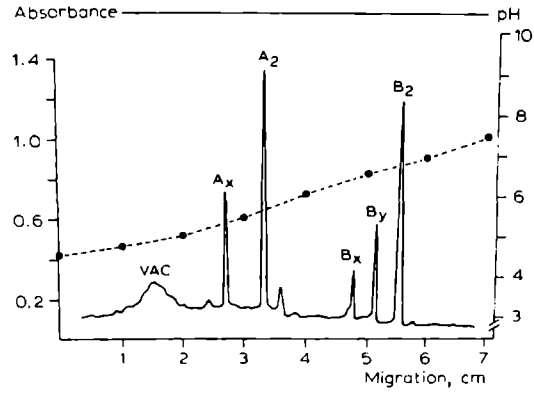

Fig. 1. Scanning profile of isoelectric focusing gel of low-M_r α-crystallin from old human lens. Absorbance was measured at 580 nm (——); the pH profile (– – –) was measured using a calibrated MI-410 microcombination pH probe.

Results and Discussion

The very acidic chains seem to be involved in the process of protein insolubilization since we isolated 2 mg from the water-soluble and 20 mg from the water-insoluble protein fraction of 100 cataractous lenses. There is an obvious reason to assume that the very acidic chains are derived from α-crystallin subunits since they are found in the low-M_r α-crystallin fraction. Isoelectric focusing in the presence of urea reveals the occurrence of the very acidic chains (fig. 1). Combining the patterns obtained for low-M_r and high-M_r crystallins isolated from nuclear cataractous lenses of the same range of age (50–90 years) leads to the following observations (fig. 2a, b): we found decreasing ratios of acidic to basic chains in low-M_r α-crystallin related to the cataractous state. The stain ratios vary from 1.9 in normal low-M_r α-crystallin to 1.3 in the most severe nuclear cataractous state (nigra nucleus). In high-M_r crystallin increasing ratios of acidic to basic chains are found related to the cataractous state (1.0–2.5). From these findings we conclude that the very acidic chains are involved in the process of protein aggregation during nuclear cataractogenesis, since the slight increase in the proportion of very acidic chains (fig. 2b) in both low-M_r and high-M_r crystallin during nuclear cataractogenesis has a strong effect on the ratios of acidic to basic chains (fig. 2a).

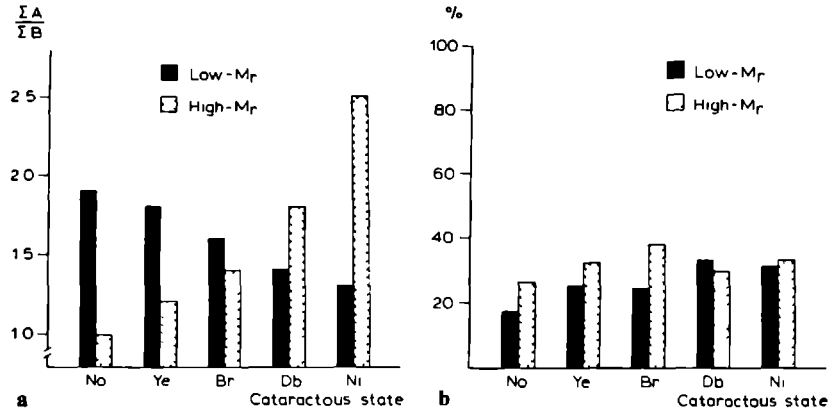

Fig. 2. Aggregation state-dependent changes in water-soluble human lens proteins during nuclear cataractogenesis. **a** Ratios of acidic [ΣA = VAC + A_x + A_2] to basic chains [ΣB = B_x + B_y + B_2] in low M_r α-crystallin and high-M_r crystallins in relation to the nuclear cataractous state. Ratios were calculated on stain basis. **b** Proportions of very acidic chains in low-M_r α-crystallin and high-M_r crystallins in relation to the cataractous state. Calculations were on stain basis and proportions were expressed as percentage of total chains [VAC/ΣA + ΣB) × 100%]. No = Normal lens; Ye = yellow nucleus; Br = brown nucleus, Db = dark brown nucleus, Ni = nigra nucleus.

An increase in the proportion of very acidic chains in low-M_r α-crystallin from 17% in normal lens to 30% in nigra cataract is accompanied by a decrease in the ratios acidic to basic chains in the same protein fraction from 1.9 in normal lens to 1.3 in nigra cataract. On the other hand, an increase in the proportion of very acidic chains in high-M_r crystallin from 26% in normal lens to more than 30% in nigra cataract is accompanied by a strong increase in the ratios acidic to basic chains in the same protein fraction from 1.0 to 2.5.

The isolated very acidic chains revealed charge and size heterogeneity as judged by ion-exchange chromatography, isoelectric focusing and SDS gel electrophoresis (fig. 3a, b). Isoelectric points and molecular weight were estimated, obtaining ranges of pI 4.6–4.7 and molecular weight 6,500–12,500. No N-terminal amino acid residues could be detected applying manual Edman sequence determination methods.

By separation of very acidic chains by high pressure ion-pairing liquid chromatography on reverse phase columns (10RP18) [2] at least 9 fractions

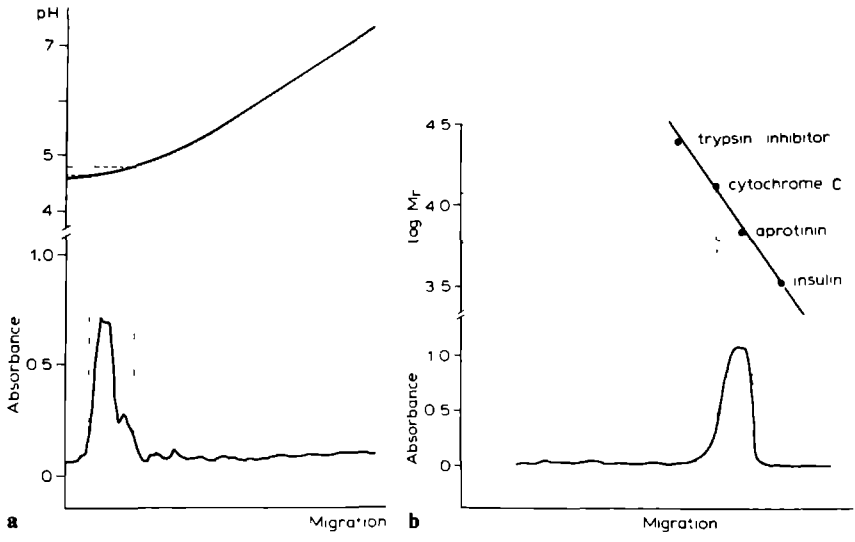

Fig. 3. Electrophoresis of very acidic chains from old human lenses. **a** Scanning profile (580 nm) and pH profile of isoelectric focusing gel of the very acidic chains. **b** Scanning profile (580 nm) of dodecyl-sulphate gel of the very acidic chains and calibration curve.

were collected, differing presumably in polarity (detailed information will be published elsewhere). Surprisingly, the isolated peptide fractions (all lacking detectable N-terminal amino acid residues) revealed a fairly constant high acidic (Asp, Glu) to basic (His, Lys, Arg) amino acids ratio, amounting to 2.5 ± 0.3 ($n = 9$).

Tryptic and chymotryptic peptide mapping of unfractionated very acidic chains revealed close resemblance between the very acidic chains and human α-A chains. Considering that the very acidic chains are found in low-M_r α-crystallin, show resemblance with α-crystallin subunits in peptide mapping and sequence analysis it seems likely that they are derived from human α-A chains.

If we plot the isoelectric points of all known degradation and deamidation products found in bovine lenses versus a parameter describing the density of excess acidic amino acid residues per unit of surface area [9] for each polypeptide we obtain a good linear correlation (fig. 4). If the very acidic chains are derived from the human α-A chain their empirically derived data

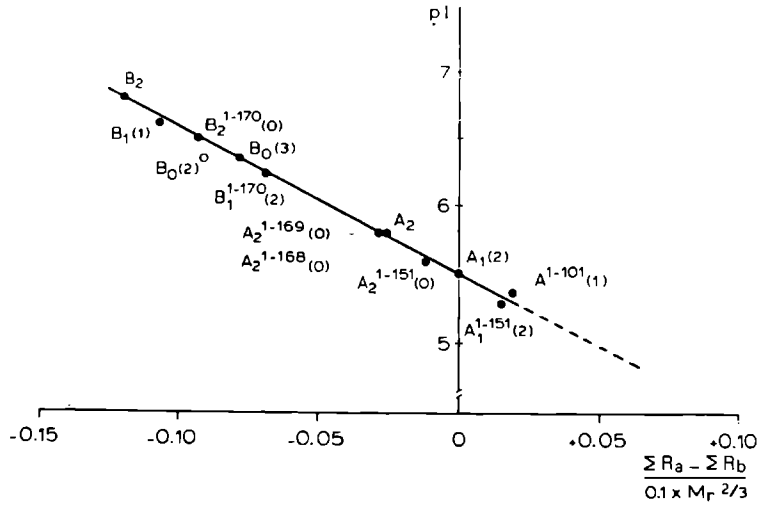

Fig. 4. Relationship between isoelectric point and density of excess acidic amino acid residues on the surface of bovine α-crystallin subunits. The number of deamidations known to occur in the corresponding sequence of the primary gene products (B_2, A_2) are given in parentheses. B_0 (3) has been tentatively derived from B_0 (2), the latter designated by (0). ΣR_a = Number of acidic residues (Asp, Glu); ΣR_b = number of basic residues (His, Lys, Arg).

should correlate with the data depicted in figure 4. Knowing the ranges of pI and molecular weight (4.6–4.7 and 6,500–12,500, respectively) one could calculate the number of deamidations occurring in such a chain to give this correlation with the data for α-crystallin subunits. The human chain corresponding to the bovine A^{1-101} chain (molecular weight 11,978) contains 4 Gln residues and one Asn residue. The latter amino acid is the C-terminal residue of the assumed degradation product derived from the human A-chain and is sometimes found deamidated [6]. Calculation of the number of deamidated residues in this chain to produce a pI range of 4.6–4.7 amounts to 3.7–4.4. Considering empirical data obtained for pentapeptides containing a possibly deamidating Gln residue [11], it is likely that the Gln residues in positions 90, 50, 25 and 6 of the human A-chain could be prone to deamidation during human life time. If these four Gln residues have equal chance to be deamidated one could expect 24 chains resembling the human A^{1-101} chain in size but differing from it in charge.

Immunochemically the very acidic chains revealed no precipitation lines with anti-sera raised against human α-crystallin Another puzzling factor is the non-tryptophan fluorescence exhibited by the very acidic chains (excitation maxima at 285 and 350 nm, emissions at 410 and 435 nm) This fluorescence could in part be due to the same components occurring in the yellow protein fraction from nuclear cataractous human lenses

Although strong indication has been presented in this paper that the very acidic chains are derived from α-crystallin A-chains and are involved in aging processes of human lens proteins the elucidation of their structure needs continuing investigation

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CHAPTER IXb

POSTSYNTHETIC PRODUCTS OF ALPHA-CRYSTALLIN IN THE AGING HUMAN EYE LENS

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9. POSTSYNTHETIC PRODUCTS OF α-CRYSTALLIN IN THE AGING HUMAN EYE LENS

Summary

Polypeptide chains with low isoelectric points, occurring in the crystallins of 30- to 80-year-old human eye lenses, were studied to determine whether they are involved in post-synthetic alterations similar to those known from bovine α-crystallin. Isoelectric focusing in the presence of 6 M urea revealed that these very acidic chains occurred in old human α-crystallin besides five major polypeptide chains; they showed a diffuse band in the pI range 4.6-4.7. By dodecyl sulfate gel electrophoresis an apparent molecular weight range from 6,500 to 12,500 was observed. Ion-exchange chromatography in the presence of urea was applied in order to obtain enriched preparations of the very acidic chains. Data concerning the primary structure of the very acidic chains were gathered by reversed phase high-pressure liquid chromatography under ion-pairing conditions, tryptic and chymotryptic peptide mapping in combination with amino acid analysis and dansyl-Edman degradation experiments. The very acidic chains showed a high degree of similarity to the A-type chains of human α-crystallin and it was concluded that they are derived from the latter by degradation, deamidation and racemization.

It was previously shown by van Kleef et al. (1974, 1975 and 1976) that the polypeptide chains of bovine α-crystallin are subject to deamidation and specific age-dependent degradation processes. Comparable events seem to take place in human α-crystallin (Roy and Spector, 1976a; Kramps et al., 1978a). Newly-synthesized bovine and, presumably, human α-crystallin is composed of two and at most three types of subunits, respectively (Roy and Spector, 1976a,b). Bovine α-crystallin is composed of 30-55 subunits (Siezen et al., 1978a), consisting of primary gene products, the acidic A_2 and the more basic B_2 chains, and various derivatives. For reasons of analogy, it seems likely that most of the subunits found in human α-crystallin are also formed by post-synthetic modifications (Kramps et al., 1978a,b; Spector et al., 1976).

In the crystallins of old human lenses polypeptide chains with relatively low isoelectric points were found. Much more of these "very acidic" chains occurred in the urea-soluble lens fraction. These chains were not found in bovine lens α-crystallin, whereas other (degraded) chains, which are present in bovine α-crystallin, were not found in human α-crystallin (Kramps et al., 1976, 1978a).

It has been suggested that human α-crystallin might be converted into high molecular weight proteins and even insoluble aggregates (Roy and Spector, 1976a). Giant protein aggregates may accumulate in old human lenses and cause lens opacification;

theoretical considerations and biochemical investigations support this hypothesis (Roy and Spector, 1976a; Benedek, 1971; Jedziniak et al., 1978b; Bettelheim, 1979). The insoluble fraction, increasing in amount on aging of the human lens, is composed of proteins derived from water-soluble precursors (Garner and Spector, 1979). Moreover, racemized amino acid residues seem to accumulate in these protein fractions on aging (Masters et al., 1978; Garner and Spector, 1978).

In order to find out which types of post-translational processes have led to the formation of the very acidic chains in old human lenses, the primary structure of the very acidic chains was studied.

MATERIALS AND METHODS

Preparation of lens fractions

Human cataractous lenses of individuals varying from 60-80 years were obtained from operations at the Eye Clinic of the Free University of Berlin. Lenses used were mainly those with a yellow nucleus. Normal human lenses were removed within 5 h after death of individuals, varying in age from 30 to 80 years, in the Eye Hospital at Rotterdam, The Netherlands. α-Crystallin and the urea-soluble fraction were isolated from decapsulated lenses as described previously (Kramps et al., 1976). α-Crystallin from bovine lenses was isolated as described by van Kleef and Hoenders (1973).

Gel electrophoresis

Isoelectric focusing in the presence of 6 M urea was performed in gels, containing 3.3% acrylamide (w/v) and 0.08% bisacrylamide (w/v) (van Kleef and Hoenders, 1973). Dodecyl sulphate gel electrophoresis was performed according to Laemmli (1970) with minor modifications (Kramps et al., 1976). Two-dimensional electrophoresis of proteins was performed by isoelectric focusing in the first and dodecyl sulphate gel electrophoresis in the second direction, according to methods described before (Kramps et al., 1978a). Isoelectric focusing gels were punctured with an MI-410 microcombination pH-probe (USA Microelectrodes Inc.) to determine pH-gradients after calibration of the probe with several buffers.

Isolation of very acidic polypeptide chains

Isolation of the very acidic chains was carried out by ion-

exchange chromatography on carboxymethyl cellulose (CM-52, Whatman). About 200 mg protein, dissolved in 70 ml of the initial buffer was applied to the CM-cellulose column (50x1.6 cm) equilibrated with 20 mM NaOAc buffer (pH 5.0) containing 7 M urea and 0.02% dithioerythritol (w/v). After washing with initial buffer, a linear gradient (2x200 ml) from 20 to 150 mM NaOAc (pH 5.0), containing 7 M urea and 0.02% dithioerythritol (w/v), was applied. Elution was carried out at 4°C at a flow rate of 15 ml/h; fractions of 3 ml were collected. The absorbance of the effluent was monitored at 278 nm with a Uvicord III absorptiometer. Selected fractions were pooled, dialyzed against water (3,500 daltons cut-off tubing), lyophilized and stored at -20°C.

Subunit analysis

Tryptic and chymotryptic hydrolyses, peptide mapping (pH 6.5) and amino acid analyses were performed as described by van der Ouderaa et al. (1973). Dansyl-Edman sequence determinations were carried out as described by de Jong et al. (1976). Carboxy-terminal amino acid sequence determination was performed according to Laine et al. (1978).

Enzymatic hydrolysis

Enzymatic hydrolysis of peptides was performed by adding 0.04 mg aminopeptidase M (Röhm and Haas) in 100 µl 0.1 M phosphate buffer (pH 7.6) to about 100 nmol peptide. After 24 h at 37°C, the mixture was diluted with 900 µl sodium citrate buffer (pH 2.2) and used directly for amino acid analysis.

High pressure liquid chromatography

Ion-pairing liquid chromatography, utilizing N-cetyl-N,N,N-trimethyl-ammoniumbromide (CTAB) in combination with varying methanol concentrations, was applied to fractionate the very acidic chains on the basis of side chain polarity differences of amino acid residues (Hancock et al., 1979; 1978, Molnar and Horvath, 1977). Samples of very acidic chains, varying between 1 and 2 mg, were injected on a reversed phase column composed of Lichrosorb-10 RP18 (Chrompack). The column (30x0.9 cm) was equilibrated with 0.1% (w/v) CTAB in deionized water (pH 7.0). Flow rates of 1.5 ml/min were maintained constant by means of a pressure drop of 7MPa. A linear gradient (2x50 ml) from 0.1% (w/v) CTAB, 0% methanol to 0.1% CTAB, 99,9% methanol (v/v) was applied. The absorbance of the effluent was monitored at 254 nm using a Pye Unicam LC3 detector, equipped with 8 µl flow-through cell. Selected fractions were pooled, methanol was removed under vacuum and fractions dialyzed against water (3,500 daltons cut-off tubing). Amino acid analysis after acid hydrolysis and enzymatic digestion of fractions eluting from the column was performed on a Chromaspek (Rank-Hilger) amino acid analyzer.

Gas chromatography of optical isomers of aspartic acid

Optical resolution of D, L-aspartic acid by gas chromatography was accomplished using methods described by Weygand et al. (1963) and Halpern and Westly (1965). 0.25 g L-proline (Sigma), dried by heating during 30 min at 110°C under vacuum, was dissolved in 1 ml trifluoroacetic anhydride (Merck). The solution was cooled to -15°C, then warmed at 30°C during 30

mir. The trifluoroacetic anhydride was evaporated under vacuum. The residue was refluxed during 30 min in 1 ml thionyl chloride (Merck) and the excess of reagent evaporated. The product was dissolved in ethyl acetate, washed with water and neutralized with triethylamine (Merck). The ethyl acetate layer was dried with solid, anhydrous sodium sulfate (Na_2SO_4) and 1-2 µl samples were analyzed on an 1.8 m x 3 mm coiled glass column packed with 3% SE-30 on Gas-Chrom Q, 100-120 mesh (Applied Science Laboratories State College, Pa.). A Varian Aerograph model 2100 chromatograph, equipped with a flame ionization detector and a linear temperature programmer, was used. The chromatographic conditions were: injector temperature, 230°C, detector temperature, 250°C, temperature program, 150-250°C at 4°C/min and finally 250°C isothermally; carrier gas (nitrogen) flow rate, 25 ml/min.

Samples for gas chromatography were hydrolyzed in 6 N HCl during 22 h at 110°C under vacuum. After hydrolysis, samples were dried overnight under vacuum over NaOH pellets. Samples of reference compounds, D- and L- aspartic acid, revealed equal molar responses.

Isoelectric focusing in the presence of urea revealed the occurrence of a group of polypeptide chains with isoelectric points below pH 5 in low molecular weight α-crystallin isolated on Bio-Gel A-5m (Fig. 9.1). After scanning the gels

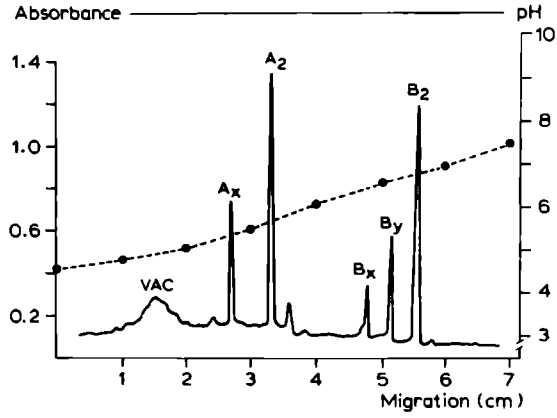

Fig. 9.1. Scanning profile of an isoelectric focusing gel of low molecular weight α-crystallin from old human lens. Absorbance was measured at 580 nm (——); the pH profile (---) was measured using a calibrated MI-410 microcombination pH-probe. VAC, very acidic chains. A_x, A_2, B_x, B_y and B_2 represent the major polypeptide chains of α-crystallin from old human lenses (Kramps et al., 1978a).

and integrating the peaks, no age-related changes were found in the proportion of very acidic chains, occurring in the α-crystallin from 30- to 80-year-old human lenses (Fig. 9.2). The ratio total acidic chains over total basic chains in low molecular weight α-crystallin revealed no age-related changes after 50 years of age (Fig. 9.2). The high molecular weight protein fraction (van Haard et al., 1978), isolated on Bio-Gel A-5m

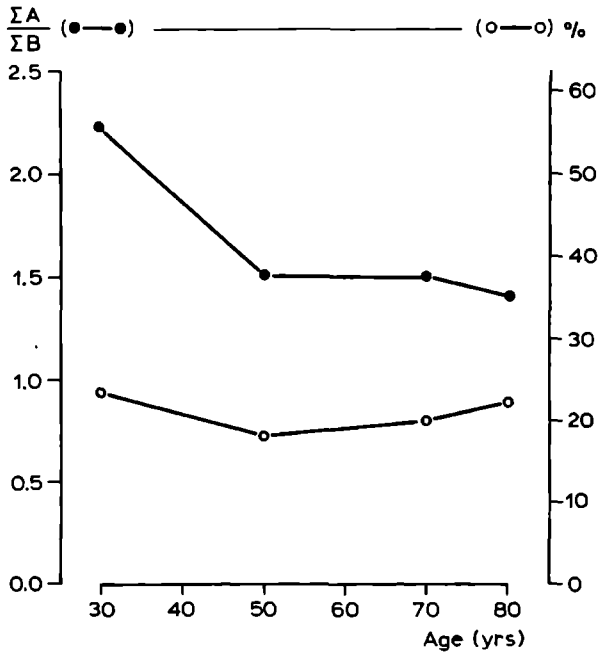

Fig. 9.2. Subunit characteristics of low molecular weight α-crystallin as a function of age.
●-●, Ratio of total acidic (ΣA = VAC + A_x + A_2) to total basic chains (ΣB = B_x + B_y + B_2), calculated on stain basis.
o-o, Proportion of very acidic chains; calculations were on stain basis and proportions were expressed as percentage of total chains (VAC/(ΣA + ΣB) x 100%).

from the water-soluble fraction (100,000 g supernatant) of the same lenses, revealed neither age-related alterations in the content of very acidic chains nor in the acidic over basic chains ratio the values obtained were roughly 24% and 2, respectively (not shown).

For further characterization of the very acidic chains, we purified them by means of ion-exchange chromatography on CM-cellulose in the presence of 7 M urea. In order to obtain rea-

sonable quantities, the column was strongly loaded. After choosing a suitable gradient, the very acidic chains could be separated from the (unresolved) other polypeptides (Fig. 9.3). Due

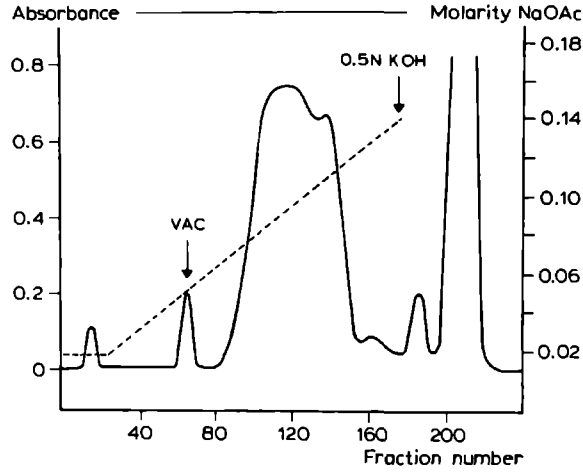

Fig. 9.3. Elution profile of human lens proteins on CM-cellulose in the presence of 7 M urea. The position of the very acidic chains (VAC) is indicated. Absorbance was measured at 280 nm.

to lack of normal lenses, yellow nuclear-cataractous lenses (Kramps et al., 1976) were pooled and processed further. We were able to isolate 2 mg very acidic chains from the water-soluble and 20 mg from the water-insoluble protein fraction of 100 cataractous lenses. The isoelectric focusing and dodecyl sulphate gel electrophoresis patterns of the very acidic chain fraction are shown in Figure 4. On dodecyl sulphate gels their molecular weights were estimated to be within the range of 6,500 and 12,500 daltons, accumulating roughly at 10,000 daltons. The very acidic chains appeared as a faint band corresponding to isoelectric points ranging from 4.6 to 4.7. The

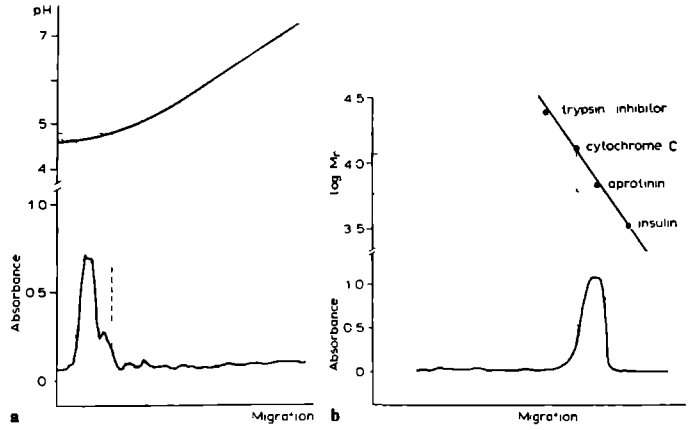

Fig. 9.4. Electrophoresis of very acidic chains from old human lenses.
a. Scanning profile (580 nm) and pH profile of isoelectric focusing gel.
b. Scanning profile (580 nm) of dodecyl sulphate gel and calibration curve.

broad bands observed after applying both electrophoretic techniques might indicate that the very acidic chains have different molecular weights but similar isoelectric points. More likely, however, the very acidic chains have different isoelectric points but almost identical molecular weights (near 10,000 daltons, see Fig. 9.4b) since it is known that some *in vitro* and *in vivo* degradation products of bovine α-crystallin with molecular weights below 12,000 daltons show broad bands or anomalous behavior on dodecyl sulphate gel electrophoresis (Siezen and Hoenders, 1979). This supposition was further supported by means of two-dimensional analysis of the very acidic chains, applying isoelectric focusing in the presence of urea in the first and dodecyl sulphate slab gel electrophoresis in the

second direction; moreover, after cutting isoelectric focusing gels into small pieces and applying each piece to dodecyl sulphate gels, it was observed that the very acidic chains still were migrating as a broad band with similar migration rate (not shown).

In an attempt to obtain better resolution of the very acidic chains, basic and acidic urea gel electrophoresis (Roy and Spector, 1976) as well as dodecyl sulphate gel electrophoresis in the presence of urea (Swank and Munkres, 1971) was performed, no improved separation or band narrowing was achieved by these methods.

Furthermore, we tried to fractionate the very acidic chains by means of ion-pairing high pressure liquid chromatography (Hancock et al., 1979, 1978; Molnar and Horvath, 1977). The elution pattern obtained revealed at least 9 fractions (Fig. 9.5).

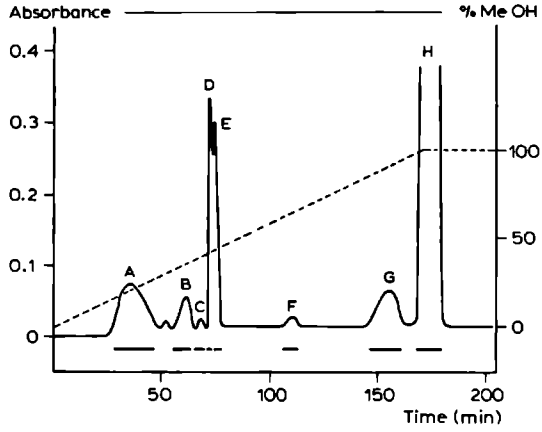

Fig. 9.5. Elution pattern of very acidic chains on a reversed phase high pressure liquid chromatography column under ion-pairing conditions.

Surprisingly, the isolated peptide fractions as well as the unfractionated material were all devoid of detectable NH_2-terminal α-amino groups and, after acid hydrolysis, showed quite similar amino acid compositions. A fairly constant high acidic (Asp, Glu) over basic (His, Lys, Arg) amino acid residue ratio was found, amounting to 2.5 ± 0.3 (S.D.) for the 9 fractions indicated in Figure 9.5.

Different retention times on reversed phase columns are reported for peptides containing racemized amino acid residues (Lundanes and Greibrokk, 1978); the optical isomers of amino acids cannot be separated on the amino acid analyzer without the addition of suitable agents (Hare and Gil-Av, 1979). Further investigation of the very acidic chains was, therefore, focused on the amino acid aspartic acid, the racemization rate of which can be used to estimate the age of living mammals and their tissues (Helfman et al., 1977; Williams and Smith, 1977). Using gas chromatography and a derivatization method involving non-racemizable L-proline coupled with each antipode (Weygand et al., 1963; Halpern and Westley, 1965), it was found that the very acidic chains revealed approximately 30% racemization of L-aspartic acid. The gas chromatographic pattern obtained for the acid hydrolyzate of the very acidic chains is depicted in Figure 9.6. It was also established that other amino acids revealed no detectable racemization.

The peptide maps of the tryptic and chymotryptic digests of the unfractionated very acidic chains showed close resemblance with those obtained from the A-chains of human α-crystallin.

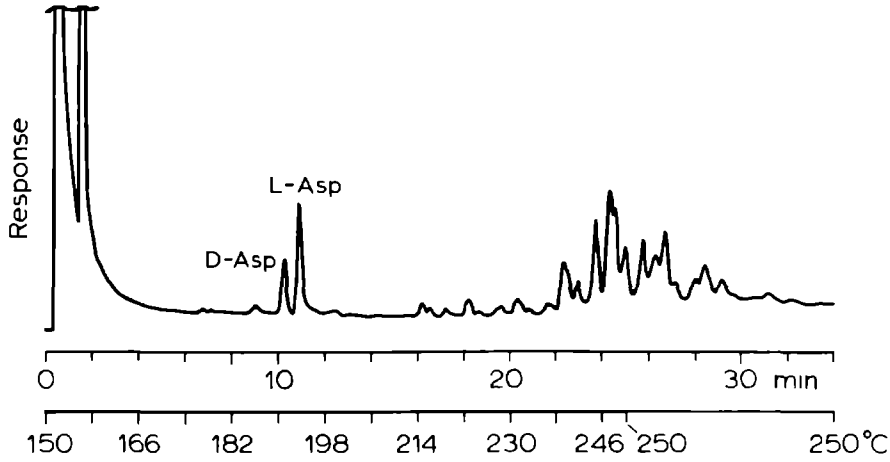

Fig. 9.6. Gas chromatographic separation of a hydrolyzate of the very acidic chains treated with N-trifluoracetyl-L-prolyl chloride reagent.

This has already been reported for the tryptic peptides by Kramps et al. (1978a), who isolated the very acidic chains by a slightly different method. The results of amino acid analyses, obtained after acid hydrolysis and aminopeptidase M digestion of peptides from the fingerprints, led to the conclusion that one peptide (T5) occurred in two differently charged forms, presumably due to deamidation of an aminoterminal glutamine residue. Tryptic peptides, which were insoluble in the electrophoresis buffer (pH 6.5), were isolated by gel chromatography on Sephadex G-50 SF (Pharmacia) as described by van der Ouderaa (1973) and turned out to be the tryptic peptides T_4 and T_9 from the human A-chain (Fig. 9.7).

Sulfhydryl group determination according to Siezen et al. (1978b), using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), revealed that the very acidic chains contained no detectable

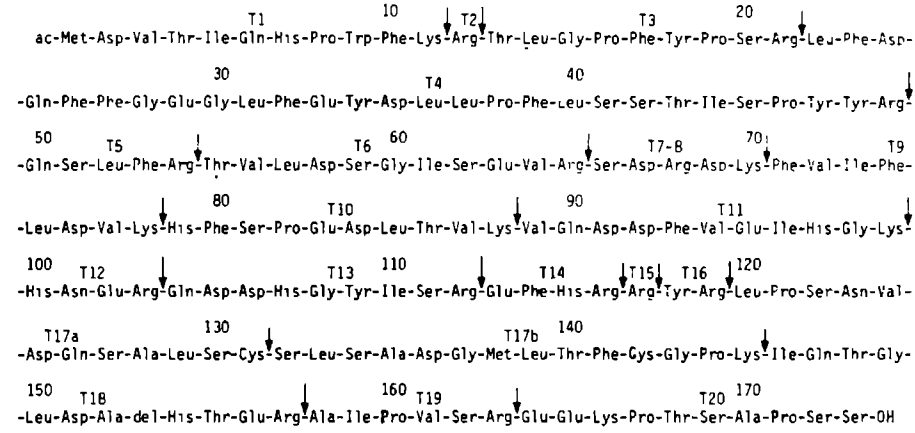

Fig. 9.7. Amino acid sequence of the A_2 chain of human α-crystallin. The points of tryptic cleavage (↓) are indicated. Residues are placed by homology with bovine A_2 chain. Taken from de Jong et al., (1975).

sulfhydryl groups.

If one plots the isoelectric points of all known degradation and deamidation products, occurring *in vivo* in bovine lens α-crystallin, versus a parameter describing the number of excess acidic amino acid residues per unit of surface area (Momany et al., 1976) for each polypeptide, a linear relationship is obtained (Fig. 9.8). Taking for granted that the very acidic chains are derived from the human αA-chain by carboxy-terminal degradation and by deamidation, their empirically obtained data should correlate with the data depicted in Figure 9.8. Knowing the ranges of isoelectric points and molecular weights of the very acidic chains (4.6-4.7 and 6,500-12,500, respectively), one can calculate the number of deamidations occurring in the corresponding A-chain sequences leading to

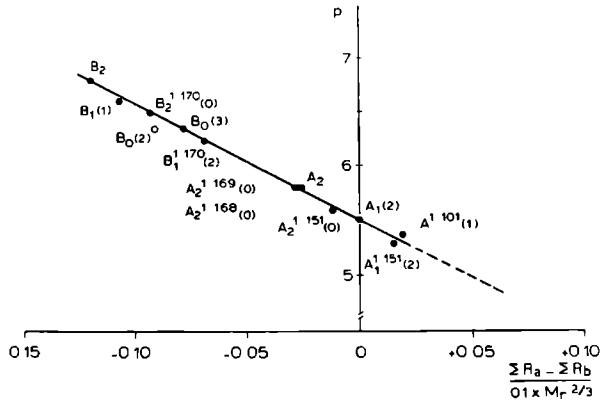

Fig. 9.8. Relationship between isoelectric points and density of excess acidic amino acid residues on the surface of bovine α-crystallin subunits. The number of deamidation known to occur in the corresponding sequence of the primary gene products (B_2, A_2) are given between brackets. B_0 (van Kleef et al., 1976) has been tentatively derived from B_0 (van Kleef et al., 1975), the latter designated by 0. ΣR_a = number of acidic residues (Asp, Glu), ΣR_b = number of basic residues (His, Lys, Arg).

the isoelectric points and molecular weight estimations. The human chain, corresponding to the *in vivo* occurring bovine A^{1-101} chain, has a molecular weight of about 12,000 daltons and contains 4 glutamine residues (positions 6, 25, 50 and 90; see Figure 9.7) and one asparagine residue (position 101). The latter amino acid (Asn^{101}) is the carboxy-terminal residue of the assumed degradation product of the human A-chain and is sometimes found deamidated in a chain named $A_2{}^{1-151}$ (Kramps et al., 1978a). The number of deamidations in the A^{1-101} chain to produce a pI range of 4.6-4.7, amounts to 3.7-4.4. Therefore, all 4 glutamine residues in the human A^{1-101} chain should be

deamidated if it were to represent the very acid chains, by means of peptide mapping and sequence analysis glutamine in posititon 50 was found to be deamidated.

Comparison of the results obtained after acid hydrolysis with those obtained after enzymatic digestion revealed that the unfractionated very acidic chains contain 2.5-3 times more glutamine than glutamic acid residues. Enzymatic hydrolysis of this material, which contains racemized amino acid residues imposes new problems as far as the quantitative interpretation of the results is concerned. With some reserve we calculated that the very acidic chains, fractionated by means of ion-pairing liquid chromatography, contain between 3 and 1.5 times more glutamine than glutamic acid residues.

Results obtained by means of carboxy-terminal analysis, using carboxy-peptidases A and B, were not reproducible.

The post-synthetic modifications of the subunits of human α-crystallin differ partly from those taking place in the subunits of bovine α-crystallin. The very acidic chains as found in human α-crystallin are not observed in bovine α-crystallin, whereas some degraded chains, which are present in bovine α-crystallin are not found in human α-crystallin (Kramps et al., 1978a). Peptide mapping of tryptic and chymotryptic hydrolyzates showed a close resemblance between the very acidic chains and certain sequences of the A-type α-crystallin subunits.

The molecular weights of the very acidic chains from old human lenses, as estimated by dodecyl sulphate gel electrophoresis, are still puzzling. If the four glutamine residues in the human A^{1-101} chain part have equal chances to be deamidated, one would expect to find 24 chains, resembling the A^{1-101} chain in size, but differing from it in charge. Since substitution of glutamine residues, e.g. due to deamidation, does not seem to influence the mobility of the chain during dodecyl sulphate electrophoresis (de Jong et al., 1978), we cannot explain the diffuse banding of the very acidic chains. Adding 7 M urea to the electrophoretic system did not lead to any improvement.

There may be at least four explanations for the diffuse banding of the very acidic chains on both isoelectric focusing as well as dodecul sulphate gels:

a. they may reveal anomalous electrophoretic behavior on dodecyl sulphate gels similar to some other *in vitro* or *in vivo*

truncated bovine α-crystallin subunits (Siezen and Hoenders, 1979),

b. in a human A^{1-101} chain, from which the very acidic chains may tentatively be derived, 10 racemizable aspartic acid residues occur and 30% of them have been shown to be racemized; it is not known whether racemization of aspartic acid residues influences the mobility of the peptide on both electrophoretic techniques applied, although it might be expected to occur on dodecyl sulphate gels considering the elution behavior of polypeptides containing racemized amino acid residues on reversed phase columns (Hancock et al., 1979; Lundanes and Greibrokk, 1978),

c. the very acidic chains reveal non-tryptophan fluorescence (van Haard et al., 1979) with excitation maxima at 285 and 350 nm and emission maxima at 410 and 435 nm, which may be attributed to components which influence the net charge or ampholyte and dodecyl sulphate binding capacity of the very acidic chains; it is known, for instance, that substitutions in mammalian α-crystallin involving proline influence the mobility of dissociated subunits on dodecyl sulphate gels due to conformational changes (de Jong et al., 1978),

d. the diffuse banding of the very acidic chains on isoelectric focusing might be explained by charge heterogeneity, due to the reported deamidations, or due to anomalous binding of carrier ampholytes to the very acidic chains as found in some proteins (Baumann and Chrambach, 1975; Dean and Messer, 1975).

It is known that the bovine A^{1-101} chain, having a molecular weight of 11,978 daltons, migrates on dodecyl sulphate gels with an apparent molecular weight of 14,000 daltons (Siezen and Hoenders, 1979). This behavior may be attributed to the carboxy-terminal part of this chain, which is rather rich in basic amino acid residues; their charge may neutralize the charge of the sulphate groups of the detergent, resulting in decreased mobility. The same phenomenon is also seen when human and bovine B-type chains are applied to dodecyl sulphate gels (Kramps, 1977; Siezen and Hoenders, 1979). Therefore, the very acidic chains may be shorter than the A^{1-101} chain part due to a process of degradation: the resulting chain might contain basic residues at the carboxy-terminus and have a molecular weight below 10,000 daltons, which is the average molecular weight as judged by dodecyl sulphate gel electrophoresis.

The processes of racemization and deamidation might influence the digestability of the very acidic chains imposing new problems in elucidating the complete set and positioning the site of events taking place in human lens proteins upon aging and cataractogenesis.

The differences in post-synthetic modifications between human and bovine α-crystallin may partially be explained by differences in age, both absolutely and relatively, since bovine lenses have a life expectancy of 30 years, whereas human lenses have one of 80 years. Species specificity might be another explanation for differences observed. As suggested earlier (van Haard et al., 1979), the very acidic chains seem to

be involved in post-synthetic processes occurring in human lens, like protein aggregation and insolubilization, leading ultimately to nuclear cataract.

SUMMARY

Since the eye lens consists mainly of structural proteins and water, it is generally accepted that cataract is a consequence of the disturbance of the structure of these proteins, the crystallins. This disturbance may lead to aggregation and precipitation of the proteins.

Most cataracts occur in men aged 50 years and older and, therefore, belong to the class of senile cataracts. In countries with a high number of solar hours, certain types of senile cataracts seem to occur at younger age of the individual; nuclear cataract, the type of cataract studied in this thesis belongs to this group.

Nuclear cataracts reveal a yellow to almost black colored and hard inner part. They can be discerned from other senile cataracts by color, localization, dimension and shape of the opacity.

The aims of this investigation are goaled at the questions how nuclear cataract arises and why this type of cataract develops at higher age in the oldest part of the lens. Because all lens components may play a role in the cataractous process and the performance of this role may depend on age, reflected by their location within the lens, the investigations were focused most of the time on different parts of the lens.

Chapter I contains a summary on aging of the lens and processes in particular those arising as a consequence of sunlight, which might play a role in the development of nuclear cataract. In a scheme, these processes are compiled and their mutual relationship is depicted. Moreover, the aims of this thesis are visualized in a model.

In chapter II, it is investigated whether the lens behaves like a visco-elastic material and whether normal and nuclear-cataractous lenses differ by compressibility. A smooth co-operation between tightly-packed proteins and the plasma membranes of the lens cells seems to be a prerequisite for maintaining lens elasticity. Lenses with an almost black nucleus turn out to be four times as hard as normal lenses of the same age, mainly due to the nuclear part. This hard consistency is not caused by a loss of water from the inner parts of the lens; therefore, it is concluded that changes occur in the hydration of proteins during aging and cataract formation. This altered water adsorption to proteins might be caused by a change in conformation of the proteins, due to processes like protein aggregation, amino acid modification and lipid association. Non-covalent protein aggregation is studied by following the appearance of water-soluble aggregates of high molecular weight. In the aggregates formed, non-protein components such as lipids occur, the origin of which will be further discussed in chapter VIII.

In nuclear-cataractous lenses a process of protein aggregation occurs, which is characterized by the development of intra-

molecular and, in a later stage, of intermolecular disulfide bridges as well as of non-disulfide covalent cross-links between polypeptide chains. The post-translational alterations mentioned are accompanied by the formation of water-insoluble, but urea-soluble fractions, which during the progress of cataract become urea-insoluble and non-reducible.

It is investigated whether in this yellow material components occur, which represent protein cross-links. Alterations in the contents of fluorescent, non-protein-bound components from different parts of the lens are described and it is questioned whether they account for the development of fluorescence in the yellow material.

Classification of lenses by color appearance is tested using data obtained by spectrophotometric determinations, performed on water-insoluble lens fractions and on different lens parts.

Chapter III deals with devising procedures to isolate lens fractions, in particular the yellow insoluble fraction and to identify cross-links occurring in this material. Although the nature of the cross-links is not yet clear, evidence is obtained that one fluorescent compound represents anthranilic acid (o-amino benzoic acid), which might be derived from tryptophan. Oxidation of specific protein residues during nuclear cataract development seems to take place according to a general principle, which is also followed by tryptophan metabolites such as kynurenine and N_1-formyl-kynurenine.

Chapter IV describes lines along which anthranilic acid may develop in lens proteins. A role of hydrogen peroxide in the cataractous process seems to become evident by the finding that anthranilic acid can be derived from tryptophan by treatment with this oxidative agent.

The fact that Pakistani lenses contain much more anthranilic acid in the yellow protein fraction than European lenses points to a relationship between sunlight and formation of hydrogen peroxide, inhibition or disappearance of reducing enzymes or, in general, loss of defense capacity of the older lens against oxidative challenge.

Chapter V contains results of investigations concerning the contents of sulfhydryl-containing components, which may play an anti-oxidative role in lens cytosol and of protein sulfhydryl groups, which are involved in stabilizing protein structure.

If lens parts are separated on the basis of cataract localization, sulfhydryl oxidation in nuclear cataractogenesis seems to be confined to the colored nuclear region. It is suggested that nuclear cataract develops from the inner nucleus towards the cortex of the lens, concomitantly with an increase in color and size. Further, it is concluded that a decrease in the concentration of glutathione is followed by an increase in oxidation of sulfhydryl groups of lens proteins, in particular in the nuclear region. Glutathione does not seem to become bound to lens proteins, which finding may

be explained by assuming a disturbance in its regenerability. Oxidation of sulfhydryl groups can be caused by the action of hydrogen peroxide and radicals, generated directly or indirectly by sunlight. The role of hydrogen peroxide is striking, since it might also be responsible for the production of anthranilic acid (chapter IV) and oxidation products of sulfur-containing protein residues (chapter VII).

Chapter VI raises doubt about the hypothesis that destruction of tryptophan residues leads to the coloration of the lens nucleus during nuclear cataractogenesis. Between lens parts with and without color no significant differences in tryptophanyl contents are found. Assigning to tryptophan a role as a radical reaction initiator, without being destructed seems to be a plausible alternative to explain nuclear coloration. Destruction of tryptophan in the cytoplasm might occur during nuclear cataract formation, but the products of such a process are hardly discernable from those of tryptophan metabolism.

In chapter VII cataractous state-related alterations are studied by Curie-point pyrolysis low voltage mass spectrometry performed on the embryonic nuclei from adult normal and nuclear-cataractous human lenses. It is shown that the relative intensities of ion signals assigned to sulfur dioxide (see also chapter III) and, tentatively, to other sulfur oxidation products correlate with the increase in lens nuclear color. Since these ion signals may represent pyrolysis fragments from methionine sulfoxide, methionine sulfone, cysteic acid and disulfide compounds, present in the parent material, it is concluded that progressive oxidation of sulfur compounds takes place during nuclear cataractogenesis. Automated pyrolysis mass spectrometry coupled with multivariate analysis of the spectral data by computer turns out to be a rapid method of characterizing submilligram samples of lens material.

The results are supported by conventional determination of sulfhydryl and tryptophan groups. No obvious decrease in the content of tryptophan and other aromatic amino acids occurs during nuclear cataractogenesis.

In chapter VIII the possible role of lipid peroxidation in the cataractous process is investigated along two lines. Firstly, it is investigated by measuring the peroxidizability of water-insoluble fractions, utilizing a carbon monoxide-hemoglobin binding test. No age-related peroxidation susceptibility is found in 25-to-90-year-old normal human lenses. Nuclear-cataractous lenses reveal no significant differences in comparison with normal lenses. Secondly, the fatty acid composition of lenses, different lens parts and water-insoluble lens fractions are determined. The proportion of unsaturated, peroxidizable fatty acids in the lipids of samples studied does not change in the age range of 55-90 years. No cataractous state-dependent changes in the proportion of individual fatty acids are observed either in the cortices or in the nuclei of nuclear-cataractous lenses. However, significant differences exist be-

tween the proportions of unsaturated fatty acids in the cortex and nucleus of each lens with the exception of the nigra lens.

Chapter IX deals with the very acidic polypeptide chains found in aging human lenses and their role in cataract formation and aging. The very acidic chains are products of post-synthetic alterations known to occur in mammalian eye lens proteins. Isoelectric focusing in the presence of urea as well as dodecyl sulfate gel electrophoresis reveal diffuse bands indicating narrow range heterogeneity in charge and molecular weight. The very acidic chains are derived from alpha-crystallin A-subunits as indicated by their primary structure data. They reveal amino acid modifications such as racemization and deamidation; in addition, the latter process has been quantitated indirectly by extrapolating data obtained from isoelectric focusing in urea and molecular weight estimation of bovine alpha-crystallin subunits to the data obtained for the very acidic chains.

SAMENVATTING

Aangezien de ooglens bijna geheel uit strukturele eiwitten en water bestaat, wordt algemeen aangenomen dat cataract of staar het gevolg is van een verstoring van de struktuur van deze eiwitten, de crystallines. Deze verstoring leidt tot aggregatie en precipitatie van de eiwitten.

De meeste cataracten komen voor bij mensen van 50 jaar en ouder en deze lenstroebelingen worden dan ook tot de seniele of ouderdomscataracten gerekend. In landen met een hoog aantal zonne-uren schijnen bepaalde vormen van seniele cataract op jongere leeftijd van het individu te ontstaan; hiertoe behoort het cataracttype dat bestudeerd wordt in dit proefschrift: de kerncataract.

Kerncataracten vertonen een geel tot bijna zwart gekleurd en hard binnenste deel. Zij onderscheiden zich in kleur, localisatie, grootte en vorm van de troebeling van andere seniele cataracten.

De doelstellingen van dit onderzoek richten zich op de vraag hoe kerncataract ontstaat en waarom deze vorm van cataract op hogere leeftijd in het oudste deel van de lens voorkomt. Aangezien alle componenten van de lens een rol kunnen spelen in dit cataracteuze proces en het vervullen van die rol mogelijk afhangt van de leeftijd, dat wil zeggen hun plaats in de lens, richtte het onderzoek zich veelal op verschillende delen van de lens.

Hoofdstuk I bevat een opsomming van de gegevens over veroudering van de lens en over processen die mogelijk een rol spelen bij het ontstaan van kerncataract, met de nadruk op de invloed van zonlicht.

In de vorm van een schema worden deze processen en hun mogelijke onderlinge relatie weergegeven. De doelstellingen van dit proefschrift worden nader aangeduid in de vorm van een model.

In hoofdstuk II wordt onderzocht of de lens zich als een viscoelastisch materiaal gedraagt en of normale en kerncataracteuze lenzen onderling verschillen in compressibiliteit. Een samenspel tussen eiwitten in hoge concentratie en de plasmembranen van de lenscellen lijkt bepalend te zijn voor handhaving van de elasticiteit van de lens. De lenzen met een bijna zwarte kern blijken bijna 4x zo moeilijk samendrukbaar te zijn als normale lenzen van dezelfde leeftijd. Deze hardheid gaat niet gepaard met een verlies aan water uit de binnenste delen van de lens, zodat geconcludeerd wordt dat veranderingen in de eiwithydratatie tijdens veroudering en cataractvorming optreden. De verandering in waterbinding door eiwitten kan te wijten zijn aan veranderingen in de conformatie van de eiwitten, die ontstaan zijn door processen als eiwitaggregatie, aminozuurmodificatie en lipideassociatie. Niet-covalente eiwitaggregatie wordt nader bestudeerd aan de hand van het ontstaan van water-oplosbare aggregaten van hoog moleculair gewicht. In de gevormde aggregaten blijken ook niet-eiwitcomponenten, zoals lipiden, voor te ko-

men, waarvan de oorsprong in hoofdstuk VIII besproken wordt.

In kerncataracteuze lenzen treedt een proces van eiwitaggregatie op dat getypeerd wordt door het ontstaan van intramoleculaire en - in een later stadium - van intermoleculaire disulfide-bruggen, naast niet-disulfide covalente bindingen (cross-links) tussen polypeptide ketens. De beschreven posttranslationele veranderingen leiden tot de vorming van wateronoplosbare, maar ureum-oplosbare fracties, die bij het voortschrijden van de cataract ureum-onoplosbaar en niet-reduceerbaar worden. Er wordt onderzocht of in dit gele materiaal eiwitcross-links voorkomen.
De veranderingen in de gehaltes aan fluorescerende, niet aan eiwit gebonden componenten in verschillende delen van de lens worden beschreven en in verband gebracht met het ontstaan van de fluorescentie in het gele materiaal.
De classificaties van lenzen op basis van de kleur wordt getoetst aan de hand van gegevens, verkregen uit spectrofotometrische bepalingen aan water-onoplosbare lensfracties en aan verschillende delen van de lens.

Hoofdstuk III behandelt procedures voor het isoleren van lensfracties, met name van de gele onoplosbare fractie en het identificeren van cross-links voorkomend in dit materiaal.

Hoewel de aard van de cross-links niet opgehelderd is, werden aanwijzingen verkregen over het bestaan van een fluorescerende component, te weten anthranilzuur (o-amino-benzoëzuur), die mogelijk is afgeleid van tryptofaan. Oxidatie van bepaalde eiwitresiduen lijkt op te treden tijdens kerncataractvorming volgens een algemeen principe, dat ook gevolgd wordt door tryptofaanmetabolieten als kynurenine en N_1-formylkynurenine.

Hoofdstuk IV beschrijft wegen waarlangs anthranilzuur kan ontstaan in lenseiwitten. De mogelijke rol van waterstofperoxide in het cataracteuze proces lijkt bevestiging te krijgen in de bevinding dat anthranilzuur kan ontstaan uit tryptofaan door behandeling met waterstofperoxide.

Het feit dat Pakistaanse lenzen beduidend meer anthranilzuur in de gele eiwitfractie bevatten dan Europese lenzen duidt op een verband tussen zonlicht en de vorming van waterstofperoxide, remming of verdwijnen van reducerende enzymen of - algemener gezegd - verlies van beschermend vermogen van de oudere lens tegen oxidatieve aanvallen.

Hoofdstuk V bevat resultaten van onderzoek naar de gehaltes aan sulfhydrylgroep-bevattende componenten die een anti-oxidatieve rol kunnen spelen in het cytosol en aan sulfhydrylgroepen van eiwitten, die betrokken zijn bij het in stand houden van de eiwitstruktuur.

Het gekleurde deel van de lens is groter naarmate de kleur donkerder bruin wordt. Uit deze bevinding wordt afgeleid dat kerncataract zich ontwikkelt in de kern en onder verdieping van de kleur naar de buitenste delen van de lens voortschrijdt. Met de uitbreiding van de cataract gaat een verlaging van de glutathionconcentratie in de kern gepaard; het aantal eiwit-

disulfide bruggen neemt toe in de richting van de embryonale kern.

Uit deze beide gegevens wordt geconcludeerd dat een afname in de bescherming van de lenseiwitten tegen oxidatie gevolgd wordt door toename van geoxideerde aminozuurresiduen. De oxidatie van de beschermende stoffen - voornamelijk glutathion - gaat niet gepaard met binding aan eiwitten; geconcludeerd moet worden dat de regeneratie van deze stoffen gestoord is.

De oxidatie van sulfhydrylgroepen kan zijn oorzaak vinden in de inwerking van radicalen en waterstofperoxide, die direkt of indirekt gegenereerd kunnen worden door zonlicht. De mogelijke rol van waterstofperoxide valt op, aangezien het ook verantwoordelijk kan zijn voor de produktie van anthranilzuur (hoofdstuk IV) en oxidatieprodukten van zwavelhoudende eiwitresiduen (hoofdstuk VII).

In hoofdstuk VI ontstaat twijfel betreffende de hypothese, dat destructie van tryptofaanresiduen leidt tot verkleuring van de lenskern tijdens kerncataract.

In de gekleurde en ongekleurde delen van de lens vertonen de eiwitten geen verschillen in tryptofaangehaltes. De rol van tryptofaanresiduen als initiatoren van radicaalreakties, zonder zelf gedestrueerd te worden, lijkt een interessant alternatief om de verkleuring van de lenskern te verklaren.

Destructie van tryptofaan treedt mogelijk wel op in het cytoplasma tijdens cataract, maar de produkten daarvan zijn moeilijk te onderscheiden van die van het tryptofaanmetabolisme.

Hoofdstuk VII beschrijft verder onderzoek naar veranderingen die optreden in cataracteuze lenzen. Dit onderzoek, verricht door middel van Curiepunt laag-voltage pyrolyse-massaspectrometrie, is uitgevoerd met embryonale kern van normale en kerncataracteuze lenzen van volwassenen. Geautomatiseerde pyrolyse-massaspectrometrie, gevolgd door multivariantieanalyse, blijkt een snelle methode te zijn waarmee het mogelijk is lensmateriaal in submilligramhoeveelheden te karakteriseren.

Met behulp van deze techniek wordt aangetoond dat de relatieve intensiteiten van ionsignalen, toegeschreven aan zwaveldioxide (zie ook hoofdstuk III) en andere oxidatieprodukten van zwavelhoudende aminozuren, een relatie vertonen met de kleur van de lenskern. Aangezien deze signalen slechts afkomstig kunnen zijn van pyrolysefragmenten van methioninesulfoxide, methioninesulfon, cysteinezuur en disulfide-verbindingen, die in het lensmateriaal aanwezig zijn, mogen wij concluderen dat gedurende kerncataractvorming een voortschrijdende oxidatie van zwavelhoudende verbindingen plaatsvindt.

De resultaten worden bevestigd door middel van conventionele bepalingen van de gehaltes aan sulfhydrylgroepen en tryptofaan. Er is geen afname in de gehaltes van tryptofaan en andere aromatische aminozuren tijdens het ontstaan van kerncataract.

In hoofdstuk VIII wordt de mogelijke rol van lipide-peroxidatie in het cataracteuze proces langs twee wegen onderzocht. Enerzijds wordt in de ouder wordende normale lens (25-90 jaar)

gekeken naar de gevoeligheid van de water-onoplosbare fracties voor lipide-peroxidatie en deze vergeleken met die van cataracteuze lenzen. Er blijkt geen leeftijdsafhankelijkheid te zijn en de cataracteuze monsters verschillen niet van de normale. Het probleem wordt anderszijds benaderd door de vetzuursamenstelling van hele lenzen, verschillende lensdelen en water-onoplosbare fracties te bepalen. De relatieve hoeveelheden onverzadigde, peroxideerbare vetzuren in de onderzochte monsters veranderen niet in het leeftijdsbereik van 55-90 jaar. In de cortices en nuclei van kerncataracteuze lenzen worden geen aan het cataractstadium-gerelateerde veranderingen in de relatieve hoeveelheden van de afzonderlijke vetzuren gevonden. Significante verschillen bestaan er tussen cortex en nucleus in in de relatieve hoeveelheden onverzadigde vetzuren van alle normale en cataracteuze lenzen, met uitzondering van de donkerste lenzen.

Hoofdstuk IX behandelt de in oude menselijke lenzen voorkomende zeer zure polypeptideketens, alsmede hun mogelijke rol bij cataractogenese en veroudering. De zeer zure polypeptideketens zijn produkten van post-translationele veranderingen die optreden in de eiwitten van zoogdierlenzen. Isoelectrische focusering in ureum en dodecylsulfaat-gel-electroforese tonen aan dat deze ketens heterogeen zijn in lading en moleculair gewicht. De zeer zure ketens zijn afgeleid van alpha-crystalline A-subeenheden, zoals blijkt uit gegevens over hun primaire struktuur. Zij vertonen aminozuurmodificaties als racemizatie en deamidatie. De mate van deamidatie kan door vergelijking met eigenschappen van runder alpha-crystalline polypeptideketens worden geschat.

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CURRICULUM VITAE

Paul M.M. van Haard was born on October 7th 1951 at Heerlen, The Netherlands.

In 1970, he graduated from the "St. Bernardinuscollege" at Heerlen, Gymnasium-β. In September of the same year he started his chemistry studies at the University of Nijmegen, The Netherlands. He obtained his Bachelor's degree in June 1973.

In January 1974 he married Henny Hustings.

In November 1976, he obtained his Doctoral degree in Biochemistry (Prof.Dr. S.L. Bonting), Pharmacology (Prof.Dr. J.M. van Rossum) and Organic Chemistry (Prof.Dr. B. Zwanenburg). In 1976 he passed the examination for Radiation Protection Officer in Radionuclide Laboratory, thus qualifying for a Government Licence at a limited level.

In October 1976, he was appointed as an assistant in the Department of Biochemistry, Faculty of Medical Science (Head: Prof.Dr. S.L. Bonting) at the University of Nijmegen, where he participated in teaching students and performed the research activities described in this thesis under the supervision of Prof.Dr. H.J. Hoenders. Before obtaining his Doctoral degree he participated in teaching Physical and Organic Chemistry.

In October 1978 his daughter Linda was born.

From January 1980, he is appointed at the Stichting Samenwerking Delftse Ziekenhuizen (SSDZ) at Delft, The Netherlands, Department of Clinical Chemistry (Head: Dr. J.A.P. Stroes).

STELLINGEN

I

Kerncataract is geen vorm van versnelde veroudering, kerncataract is inherent aan veroudering en haar voorkomen wordt bepaald door extrinsieke factoren.

Dit proefschrift.

II

Bij het weergeven van cataractincidentie in wetenschappelijke publicaties dient zorgvuldig te worden omgesprongen met het "inkleuren" van diagrammen, opdat geen verwarring met de huidskleur van de onderzochte patiënten optreedt.

Heyningen, R. van (1975). Scientific American 233, p. 81.

Zigman, S., Datiles, M. and Torczynski, E. (1979). Invest. Ophthalmol. 18, p. 463.

III

Het feit dat de rat - een nachtdier - een verwaarloosbare en de mens een relatief hoge ascorbinezuurconcentratie in het oogkamervocht vertoont, moet leiden tot nader onderzoek naar de invloed van de zon op de lenstransparantie bij de mens en de beschermende rol van ascorbinezuur (vitamine C) daarbij.

Varma, S.D., Kumar, S. and Richards, R.D. (1979). Proc. Natl. Acad. Sci. USA 76, 3504-3506.

IV

Het ontbreken van lysine-gehaltes in tabellen, weergevende de aminozuursamenstelling van Afrikaanse lenscrystallines is een reden de Nederlandse ontwikkelingshulp uit te breiden met de leverantie van aminozuuranalysatoren.

Alao, J.F. (1978). African J. Med. Sci. 7, 49-56.

V

De conclusies van Truscott en Augusteyn (1977) en die van Anderson, Wright en Spector (1979) staan lijnrecht tegenover elkaar waar het de afname in de gehaltes aan glutathion in lensdelen betreft. Afgaande op het aantal lenzen met eenduidige kerncataract, onderzocht door de tweede groep auteurs (2 exemplaren) is men geneigd de conclusies van de eerste groep meer vertrouwen te schenken.

Truscott, R.J.W. and Augusteyn, R.C. (1977). Exp. Eye Res. 25, 139-148.

Anderson, E.I., Wright, D.D. and Spector, A. (1979). Exp. Eye Res. 29, 233-243.

VI

De bewering van Truscott en Augusteyn (1977) en de bevestiging daarvan door Houghton en Li (1979) dat sulfhydrylgroepen in staat zijn methioninesulfoxide te reduceren tot methionine staat in schril contrast tot het gebruik van sulfhydrylgroep bevattende agentia bij het isoleren van methioninesulfoxide bevattende lenseiwitten door de eerstgenoemde auteurs.

Truscott, R.J.W. and Augusteyn, R.C. (1977). Biochim. Biophys. Acta 492, 43-52.

Houghton, R.A. and Li, C.H. (1979). Anal. Biochem. 98, 36-46.

VII

Het door het weekblad Privé aanbevolen middel tegen slapeloosheid, L-tryptofaan, leidt bij deprivatie tot debiliteit. In afwachting van verregaand wetenschappelijk onderzoek is het ethisch verantwoord dePrivéatie toe te passen bij slapeloosheid.

Weekblad Privé (1979). Nummers 15, 20, 21, 23.

Gottfries, C.G. (1978) in Sleep Research; Proc. Eur. Symp. on Sleep Research (Priest, R.G., Pletscher, A. and Ward, J., eds.), p. 178, Basle, 1978.

Bunce, G.E., Hess, J.L. and Fillnow, G.M. (1978). Exp. Eye Res. 26, 399-405.

VIII

De verbreking van disulfide-bruggen in ontvouwd β-trypsine door bestraling met licht van 309 nm bij pH hoger dan 9, zonder vorming van thiolgroepen en zonder destructie van tyrosine en tryptofaan, kan te wijten zijn aan waterstofperoxide.
Ramachandran, N. and Ghiron, C.A. Photochem. Photobiol. 29, 67-70.

IX

De tijd zal ons leren of BOM-vrouwen en BOV-mannen BOK-kinderen krijgen.
Verklaring der afkortingen: BOM = bewust ongehuwde moeder;
BOV = bewust ongehuwde vader;
BOK = bewust opgevoed/onbehouwen kind.

X

Nederland wordt een land van studenten; zelfs in de file gaat het studeren met horten en stoten vooruit.

XI

Het is niet meer aannemelijk dat het blad met de Stellingen, letterlijk, maar niet inhoudelijk het enige onderdeel van een academisch proefschrift vormt dat eruit komt rollen.

XII

De in zwang gekomen Toelichting bij een academisch proefschrift is vergelijkbaar met de toelichting bij een belastingaangiftebiljet; men heeft in beide gevallen een deskundige nodig om ze te begrijpen.

XIII

Het feit dat cichliden "schaterend van het lachen" wegzwemmen, zodra de aquariaan de vangklok in het water duwt, is geen doorslaggevend argument het gebruik van dit aquaristiek instrument af te raden.
Van Bohemen, J. (1979). Moderne aquariumtechniek voor het tropisch zoetwater aquarium. Pp. 205-206, Elsevier, Amsterdam.

Stellingen bij het proefschrift: Oxidative Challenge to Aging Human Lens leading to Nuclear Cataract.

P.M.M. van Haard

Nijmegen, 18 september 1980